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DNA POLYMERASE COMPOSITIONS FOR QUANTITATIVE PCR AND METHODS THEREOF

RELATED APPLICATIONS

The present application claims priority under 35 U.S.C. §120 as a continuation in part of U.S. Patent Application with Serial No. 10/408,601, filed April 7, 2003, which is a continuation in part of U.S. Application Serial No. 10/298,680, filed November 18, 2002, which is a continuation in part of U.S. Application Serial No. 10/280,962, Filed October 25, 2002. The entirety of each of the above applications is hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates to mutant Archaeal DNA polymerases with deficient 3'-5' exonuclease activity and/or reduced base analog detection activity, and the uses thereof.

BACKGROUND

DNA polymerases synthesize DNA molecules in the 5' to 3' direction from deoxynucleoside triphosphates (nucleotides) using a complementary template DNA strand and a primer by successively adding nucleotides to the free 3'-hydroxyl group of the growing strand. The template strand determines the order of addition of nucleotides via Watson-Crick base pairing. In cells, DNA polymerases are involved in DNA repair synthesis and replication (Kornberg, 1974, In DNA Synthesis. W. H. Freeman, San Francisco).

Archaeal DNA polymerases have a 3' to 5' exonuclease activity and a DNA synthesis activity. Many molecular cloning techniques and protocols involve the synthesis of DNA in *in vitro* reactions catalyzed by DNA polymerases. Sometimes, mutant forms of DNA polymerases are desired for particular uses. For example, DNA polymerases are used in DNA labelling and DNA sequencing reactions, using either 35S-, 32P- or 33P-labelled nucleotides. Most of these enzymes require a template and primer, and synthesize a product whose

sequence is complementary to that of the template. The 5' to 3' exonuclease activity of An Archaeal DNA polymerases is often troublesome in these reactions because it degrades the 5' terminus of primers that are bound to the DNA templates and removes 5' phosphates from the termini of DNA fragments that are to be used as substrates for ligation. The use of DNA polymerase for these labelling and sequencing reactions thus may depend upon the removal of the 5' to 3' exonuclease activity.

DNA processivity is performed by heat denaturation of a DNA template containing the target sequence, annealing of a primer to the DNA strand and extension of the annealed primer with a DNA polymerase. The concept of net DNA processivity is the ratio of DNA synthesis activity versus 3'-5' exonuclease activity (for reviews, see, e.g., Kelman et al., 1998 *Processivity of DNA polymerases: two mechanisms, one goal.* Structure 6(2):121-5; Wyman and Botchan, 1995, *DNA replication. A familiar ring to DNA polymerase processivity.* Curr Biol. 5(4):334-7; and Von Hippel et al., 1994, *On the processivity of polymerases.* Ann N Y Acad Sci. 726:118-31). DNA synthesis activity acts to polymerize nucleotides while 3'-5' exonuclease has an editing or proof-reading function to enhance the fidelity of the synthesis. Thus highly efficient DNA synthesis is generally achieved at the expense of high fidelity and vice versa. The 3'-to-5' exonuclease activity of many DNA polymerases may, therefore, be disadvantageous in situations where one is trying to achieve net synthesis of DNA and/or where fidelity is not of primary concern.

Archaeal family B DNA polymerases are uniquely able to recognize unrepaired uracil in a template strand and stall polymerization upstream of the lesion, thereby preventing the irreversible fixation of an G-C to A-T mutation (Fogg et al., 2002, Nat Struct Biol. 9(12):922-7). Uracil detection is thought to represent the first step in a pathway to repair DNA cytosine deamination (dCMP→dUMP) in archaea (Greagg et al, 1999, PNAS USA, 96:9405). Stalling of DNA synthesis opposite uracil has significant implications for high-fidelity PCR amplification with Archaeal DNA polymerases. Techniques requiring dUTP (e.g., dUTP/UDG decontamination methods, Longo et al. 1990, Gene, 93:125) or uracil-containing oligonucleotides can not be performed with proofreading DNA polymerases (Slupphaug et al. 1993, Anal. Biochem., 211:164; Sakaguchi et al. 1996, Biotechniques, 21:368). But more

importantly, uracil stalling has been shown to compromise the performance of Archaeal DNA polymerases under standard PCR conditions (Hogrefe et al. 2002, PNAS USA, 99:596).

During PCR amplification, a small amount of dCTP undergoes deamination to dUTP (%dUTP varies with cycling time), and is subsequently incorporated by Archaeal DNA polymerases. Once incorporated, uracil-containing DNA inhibits Archaeal DNA polymerases, limiting their efficiency. We found that adding a thermostable dUTPase (dUTP → dUMP + PP_i) to amplification reactions carried out with Pfu, KOD, Vent, and Deep Vent DNA polymerases significantly increases PCR product yields by preventing dUTP incorporation (Hogrefe et al. 2002, Supra). Moreover, the target-length capability of Pfu DNA polymerase is dramatically improved in the presence of dUTPase (from <2kb to 14kb), indicating that uracil poisoning severely limits long-range PCR due to the use of prolonged extension times (1-2 min per kb @ 72°C) that promote dUTP formation.

In addition to dUTP incorporation, uracil may also arise as a result of cytosine deamination in template DNA. The extent to which cytosine deamination occurs during temperature cycling has not been determined; however, any uracil generated would presumably impair the PCR performance of Archaeal DNA polymerases. Uracil arising from cytosine deamination in template DNA is unaffected by adding dUTPase, which only prevents incorporation of dUTP (created by dCTP deamination). Adding enzymes such as uracil DNA glycosylase (UGD), which excise uracil from the sugar backbone of DNA, or mismatch-specific UDGs (MUG), which additionally excise G:T mismatches, is one way to eliminate template uracil that impedes polymerization.

Alternatively, the problem of uracil stalling may be overcome by introducing mutations or deletions in Archaeal DNA polymerases that reduce, or ideally, eliminate uracil detection, and therefore, allow synthesis to continue opposite incorporated uracil (non-mutagenic uracil) and deaminated cytosine (pro-mutagenic uracil). Such mutants would be expected to produce higher product yields and amplify longer targets compared to wild type Archaeal DNA polymerases. Moreover, mutants that lack uracil detection should be compatible with dUTP/UNG decontamination methods employed in real-time Q-PCR.

It is sometimes desired for a DNA polymerase or a reverse transcriptase to have a high processivity. Processivity is a measurement of the ability of a DNA polymerase to incorporate one or more deoxynucleotides into a primer template molecule without the DNA polymerase dissociating from that molecule. DNA polymerases having low processivity, such as the Klenow fragment of DNA polymerase I of E. coli, will dissociate after about 5-40 nucleotides are incorporated on average. Other polymerases, such as T7 DNA polymerase in the presence of thioredoxin, are able to incorporate many thousands of nucleotides prior to dissociating. In the absence of thioredoxin such a T7 DNA polymerase has a much lower processivity. Processivity factors have been identified to increase the processivity of a DNA polymerase (e.g., see Carson DR, Christman MF. 2001, Proc Natl Acad Sci U S A. 98(15):8270-5).

U.S. patent 5,972,603 teaches a chimeric DNA polymerase having a DNA polymerase domain and a processivity factor binding domain not naturally associated with the DNA polymerase domain, where the processivity factor binding domain binds thioredoxin.

U.S. Patent Application with Serial No. 2002/0119467 describes a method for increasing the processivity of reverse transcriptase (RT) E. coli DNA polymerase and T7 DNA polymerase using a polynucleotide binding protein such as Ncp7, recA, SSB and T4gp32.

There is therefore a need for thermostable DNA polymerases that can amplify DNA in the presence of dUTP without compromising proofreading or polymerization activity and efficiency. There is also a need for thermostable DNA polymerases that can amplify DNA efficiently without the proof checking function of 3'-5' exonuclease activity so that the thermostable DNA polymerase exhibits increased processivity.

SUMMARY OF THE INVENTION

The present invention provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in exoI motif and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

The present invention provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in exoII motif and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

The present invention also provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in exo III motif and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

The present invention further provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in each of exo I and exo III motifs and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

In addition, the present invention provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in each of exo II and exo III motifs and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

The present invention provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in each of exo I and exoII motifs and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

The present invention provides an Archaeal DNA polymerase comprising an amino acid sequence selected from SEQ ID NOs. 83-108, and further comprising at lease one amino acid mutation in each of exoI, exo II, and exoIII motifs and another amino acid mutation at V93, where the Archaeal DNA polymerase is deficient in 3'-5' exonuclease activity.

Preferably, the mutant Archaeal DNA polymerase of the present invention is selected from the group consisting of: KOD, Pfu, and JDF-3 DNA polymerase.

Also preferably, the mutation at position V93, is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution, a Valine to Glutamine substitution, or a Valine to Asparagine substitution.

Prefarably, the mutation in exo I motif is selected from the group consisting of: aspartic acid (D) to threonine (T), aspartic acid (D) to alanine (A) and glutamic acid (E) to alanine (A).

The present invention provides an isolated polynucleotide comprising a nucleotide sequence encoding a mutant Archaeal DNA polymerase of the present invention as described above.

The present invention provides a composition comprising a mutant Archaeal DNA polymerase as described above.

Preferably, the composition of the present invention also contains an enzyme with reverse transcriptase activity.

The present invention provides a kit comprising a mutant Archaeal DNA polymerase as described above and packaging material therefor.

The kit may further contain an enzyme with reverse transcriptase activity.

Preferably, the enzyme with reverse transcriptase is a second mutant DNA polymerase.

More preferably, the enzyme with reverse transcriptase is the mutant Archaeal DNA polymerase which contains an increased reverse transcriptase activity.

The composition or kit of the present invention may further comprise a PCR additive.

The present invention provides a method for DNA synthesis comprising: (a) providing a mutant Archaeal DNA polymerase; and (b) contacting the mutant Archaeal DNA polymerase with a polynucleotide template to permit DNA synthesis.

The present invention further provides a method for determining the abundance of a polynucleotide template, comprising (a) providing a mutant Archaeal DNA polymerase; (b) contacting the mutant Archaeal DNA polymerase with the polynucleotide template to produce amplified product; and (c) determining the abundance of the amplified product, where the abundance of the amplified product is indicative of the abundance of the polynucleotide template.

Preferably, the polynucleotide template is a RNA molecule, and where the RNA molecule is reverse transcribed into cDNA before the contacting step (b).

Also preferably, the RNA is reverse transcribed by an enzyme with reverse transcriptase activity.

More preferably, the RNA is reverse transcribed by the mutant Archaeal DNA polymerase which also contains an increased reverse transcriptase activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Oligonucleotide Primers for QuikChange Mutagenesis (SEQ ID Nos: 6-14, 43-55) according to one embodiment of the invention.

Figure 2: (a) dUTP incorporation of V93E and V93R exo- mutants compared to wild type Pfu DNA polymerase according to one embodiment of the invention.

(b) PCR Amplification of Pfu V93R exo- mutant extract in the presence of 100% dUTP according to one embodiment of the invention.

Figure 3: Protein concentration, unit concentration, and specific activity of the purified Pfu V93R and V93E exo- mutants according to one embodiment of the invention.

Figure 4: Comparison of the efficacy of PCR amplification of Pfu DNA polymerase mutants and wt enzyme in the presence of different TTP:dUTP concentration ratios.

Figure 5: Comparison of the efficacy of "long" PCR amplification of Pfu DNA polymerase mutants and wt enzyme.

Figure 6: 6A. DNA sequence of example mutant Archaeal DNA polymerases according to one embodiment of the invention.

6B. Amino acid sequence of example mutant Archaeal DNA polymerases according to one embodiment of the invention

6C. DNA and Amino acid sequence of mutant Tgo DNA polymerase according to one embodiment of the invention

Figure 7: 7A. Amino acid sequence of example wild type DNA polymerase according to one embodiment of the invention (SEQ ID NOs. 83-108).

7B. Amino acid sequence alignment of example wild-type Archaeal DNA polymerases according to one embodiment of the invention.

Figure 8: dUTP incorporation of Pfu mutants compared to wild type Pfu DNA polymerase according to one embodiment of the invention.

8A. dUTP incorporation of Pfu mutants V93W, V93Y, V93M, V93K and V93R compared to wild type Pfu DNA polymerase

8B. dUTP incorporation of the Pfu V93D and V93R mutants compared to wild type Pfu DNA polymerase.

8C. dUTP incorporation of the Pfu V93N and V93G mutant compared to wild type Pfu DNA polymerase

Figure 9: DNA polymerase activity of N-terminal Pfu DNA polymerase truncation mutants according to one embodiment of the invention.

Figure 10: Oligonucleotide Primers for QuikChange Mutagenesis (SEQ ID Nos: 56-74).

Figure 11: DNA polymerase activity of KOD V93 exo- polymerase mutants according to one embodiment of the invention.

Figure 12: DNA polymerase activity of Tgo V93 exo- DNA polymerase mutants and comparison with JDF-3 V93 exo- polymerase mutants according to one embodiment of the invention.

Figure 13: DNA polymerase activity of JDF-3 polymerase mutants according to one embodiment of the invention.

Figure 14: DNA polymerase activity of Pfu polymerase deletion mutants according to one embodiment of the invention.

Figure 15: An amplification plot for comparison of three polymerases in RT-QPCR according to one embodiment of the invention.

Figure 16: A semi-log amplification plot comparing Pfu V93R and Pfu V93R exo- QPCR according to one embodiment of the invention.

Figure 17: An amplification plot comparing Pfu V93R and other DNA polymerase in multiplexing QPCR according to one embodiment of the invention.

DETAILED DESCRIPTION

DEFINITIONS

The invention contemplates A mutant DNA polymerase that exhibits deficient 3'-5' exonuclease activity and/or reduced base analog detection (for example, reduced detection of a particular base analog such as uracil or inosine or reduced detection of at least two base analogs).

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, N.Y).

As used herein, "Archaeal" DNA polymerase refers to DNA polymerases that belong to either the Family B/pol I-type group (e.g., Pfu, KOD, Pfx, Vent, Deep Vent, Tgo, Pwo) or the pol II group (e.g., Pyrococcus furiosus DP1/DP2 2-subunit DNA polymerase). In one embodiment, "Archaeal" DNA polymerase refers to thermostable Archaeal DNA polymerases (PCR-able) and include, but are not limited to, DNA polymerases isolated from Pyrococcus species (furiosus, species GB-D, woesii, abysii, horikoshii), Thermococcus species (kodakaraensis KOD1, litoralis, species 9 degrees North-7, species JDF-3, gorgonarius), Pyrodictium occultum, and Archaeoglobus fulgidus. It is estimated that suitable archaea would exhibit maximal growth temperatures of >80-85^{0}C or optimal growth temperatures of >70-80^{0}C. Appropriate PCR enzymes from the Archaeal pol I DNA polymerase group are commercially available, including Pfu (Stratagene), KOD (Toyobo), Pfx (Life Technologies, Inc.), Vent (New England BioLabs), Deep Vent (New England BioLabs), Tgo (Roche), and Pwo (Roche). Additional archaea related to those listed above are described in the following references: Archaea: A Laboratory Manual (Robb, F.T. and Place, A.R., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995

As used herein, "mutant" polymerase refers to an Archaeal DNA polymerase, as defined herein, comprising one or more mutations that alter one or more activities of the DNA polymerase, for example, DNA polymerization, 3'-5' exonuclease activity or base analog detection activities. In one embodiment, the "mutant" polymerase of the invention refers to a DNA polymerase containing one or more mutations that reduce one or more base analog detection activities of the DNA polymerase. In a preferred embodiment, the "mutant" polymerase of the invention has a reduced uracil detection activity. In a preferred embodiment, the "mutant" polymerase of the invention has a reduced inosine detection

activity. In another preferred embodiment, the "mutant" polymerase of the invention has a reduced uracil and inosine detection activity. A "mutant" polymerase as defined herein, includes a polymerase comprising one or more amino acid substitutions, one or more amino acid insertions, a truncation or an internal deletion.

A "mutant" polymerase as defined herein also includes a chimeric polymerase wherein any of the single, double or triple mutant Archaeal DNA polymerases described herein, any mutant Archaeal DNA polymerases comprising an insertion, described herein, or any of the truncated, or deleted mutant Archaeal DNA polymerases described herein, occur in combination with a polypeptide that increases processivity, thereby forming a chimera, as defined herein. A polypeptide that increases processivity is described in U.S. Patent Application with Serial No. 10/408,601, WO 01/92501 A1 and Pavlov et al., 2002, Proc. Natl. Acad. Sci. USA, 99:13510-13515, herein incorporated by reference in their entirety.

A "chimera" as defined herein, is a fusion of a first amino acid sequence (protein) comprising a wild type or mutant ARCHAEAL DNA polymerase of the invention, joined to a second amino acid sequence defining a polypeptide that increases processivity, wherein the first and second amino acids are not found in the same relationship in nature. A "chimera" according to the invention contains two or more amino acid sequences (for example a sequence encoding a wild type or mutant ARCHAEAL DNA polymerase and a polypeptide that increases processivity) from unrelated proteins, joined to form a new functional protein. A chimera of the invention may present a foreign polypeptide which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. The invention encompasses chimeras wherein the polypeptide that increases processivity and/or efficiency is joined N-terminally or C-terminally to a wild-type Archaeal DNA polymerase or to any of the mutant Archaeal DNA polymerases described herein.

As used herein, "joined" refers to any method known in the art for functionally connecting polypeptide domains, including without limitation recombinant fusion with or without intervening domains, intein-mediated fusion, non-covalent association, and covalent

bonding, including disulfide bonding, hydrogen bonding, electrostatic bonding, and conformational bonding.

As used herein, "mutation" refers to a change introduced into a wild type DNA sequence that changes the amino acid sequence encoded by the DNA, including, but not limited to, substitutions, insertions, deletions or truncations. The consequences of a mutation include, but are not limited to, the creation of a new character, property, function, or trait not found in the protein encoded by the parental DNA, including, but not limited to, N terminal truncation, C terminal truncation or chemical modification. A "mutation," according to the present invention, may be created by genetic modification or chemical modification.

As used herein, "corresponding" refers to sequence similarity in a comparison of two or more nucleic acids or polypeptides, where functionally equivalent domains or sub-sequences are identified; such functionally equivalent domains or sub-sequences or amino acids within such a domain or sub-sequence are said to "correspond". That is, two or more sequences are compared through a comparative alignment analysis in which an entire sequence is examined for regions of sequence that are similar or identical, and thus regions likely to be functionally equivalent to regions from the other sequence(s) are identified.

As used herein in reference to comparisons of an amino acid, amino acid sequence, or protein domain, the term "similar" refers to amino acids or domains that although not identical, represent "conservative" differences. By "conservative" is meant that the differing amino acid has like characteristics with the amino acid in the corresponding or reference sequence. Typical conservative substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. In calculating the degree (most often as a percentage) of similarity between two polypeptide sequences, one considers the number of positions at which identity or similarity is observed between corresponding amino acid residues in the two polypeptide sequences in relation to the entire lengths of the two molecules being compared.

As used herein, the term "functionally equivalent" means that a given motif, region, or amino acid within a motif or region performs the same function with regard to the overall

function of the enzyme as a motif, region or amino acid within a motif or region performs in another enzyme.

As used herein, "3' to 5' exonuclease deficient" or "3' to 5' exo-" refers to an enzyme that substantially lacks the ability to remove incorporated nucleotides from the 3' end of a DNA polymer. DNA polymerase exonuclease activities, such as the 3' to 5' exonuclease activity exemplified by members of the Family B polymerases, can be lost through mutation, yielding an exonuclease-deficient polymerase. As used herein, a DNA polymerase that is deficient in 3' to 5' exonuclease activity substantially lacks 3' to 5' exonuclease activity. "Substantially lacks" encompasses a complete lack of activity, for example, 0.03%, 0.05%, 0.1%, 1%, 5%, 10%, 20% or even up to 50% of the exonuclease activity relative to the parental enzyme. Methods used to generate and characterize 3'-5' exonuclease DNA polymerases including the D141A and E143A mutations as well as other mutations that reduce or eliminate 3'-5' exonuclease activity are disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000). Additional mutations that reduce or eliminate 3' to 5' exonuclease activity are known in the art and contemplated herein.

As used herein, "base analogs" refer to bases that have undergone a chemical modification as a result of the elevated temperatures required for PCR reactions. In a preferred embodiment, "base analog" refers to uracil that is generated by deamination of cytosine. In another preferred embodiment, "base analog" refers to inosine that is generated by deamination of adenine.

As used herein, "reduced base analog detection" refers to a DNA polymerase with a reduced ability to recognize a base analog, for example, uracil or inosine, present in a DNA template. In this context, mutant DNA polymerase with "reduced" base analog detection activity is a DNA polymerase mutant having a base analog detection activity which is lower than that of the wild-type enzyme, i.e., having less than 10% (e.g., less than 8%, 6%, 4%, 2% or less than 1%) of the base analog detection activity of that of the wild-type enzyme. base analog detection activity may be determined according to the assays similar to those described for the detection of DNA polymerases having a reduced uracil detection as described in

Greagg et al. (1999) Proc. Natl. Acad. Sci. 96, 9045-9050 and Example 3. Alternatively, "reduced" base analog detection refers to a mutant DNA polymerase with a reduced ability to recognize a base analog, the "reduced" recognition of a base analog being evident by an increase in the amount of >10Kb PCR of at least 10%, preferably 50%, more preferably 90%, most preferably 99% or more, as compared to a wild type DNA polymerase without a reduced base analog detection activity. The amount of a > 10Kb PCR product is measured either by spectorophotometer-absorbance assays of gel eluted > 10Kb PCR DNA product or by fluorometric analysis of > 10Kb PCR products in an ethidium bromide stained agarose electrophoresis gel using, for example, a Molecular Dynamics (MD) FluorImager™ (Amersham Biosciences, catalogue #63-0007- 79).

As used herein, "reduced uracil detection" refers to a DNA polymerase with a reduced ability to recognize a uracil base present in a DNA template. In this context, mutant DNA polymerase with "reduced" uracil detection activity is a DNA polymerase mutant having a uracil detection activity which is lower than that of the wild-type enzyme, i.e., having less than 10% (e.g., less than 8%, 6%, 4%, 2% or less than 1%) of the uracil detection activity of that of the wild-type enzyme. Uracil detection activity may be determined according to the assays described in Greagg et al. (1999) Proc. Natl. Acad. Sci. 96, 9045-9050 and as described herein below. Alternatively, "reduced" uracil detection refers to a mutant DNA polymerase with a reduced ability to recognize uracil, the "reduced" recognition of uracil being evident by an increase in the amount of >10Kb PCR of at least 10%, preferably 50%, more preferably 90%, most preferably 99% or more, as compared to a wild type DNA polymerase without a reduced uracil detection activity. The amount of a > 10Kb PCR product is measured either by spectorophotometer-absorbance assays of gel eluted > 10Kb PCR DNA product or by fluorometric analysis of > 10Kb PCR products in an ethidium bromide stained agarose electrophoresis gel using, for example, a Molecular Dynamics (MD) FluorImager™ (Amersham Biosciences, catalogue #63-0007- 79).

As used herein, the terms "reverse transcription activity" and "reverse transcriptase activity" are used interchangeably to refer to the ability of an enzyme (e.g., a reverse transcriptase or a DNA polymerase) to synthesize a DNA strand (i.e., cDNA) utilizing an RNA strand as a template. Methods for measuring RT activity are provided in the examples

herein below and also are well known in the art. For example, the Quan-T-RT assay system is commercially available from Amersham (Arlington Heights, Ill.) and is described in Bosworth, et al., Nature 1989, 341:167-168.

As used herein, the term "increased reverse transcriptase activity" refers to the level of reverse transcriptase activity of a mutant enzyme (e.g., a DNA polymerase) as compared to its wild-type form. A mutant enzyme is said to have an "increased reverse transcriptase activity" if the level of its reverse transcriptase activity (as measured by methods described herein or known in the art) is at least 20% or more than its wild-type form, for example, at least 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more or at least 2-fold, 3-fold, 4-fold, 5-fold, or 10-fold or more.

As used herein, "synthesis" refers to any in vitro method for making new strand of polynucleotide or elongating existing polynucleotide (i.e., DNA or RNA) in a template dependent manner. Synthesis, according to the invention, includes amplification, which increases the number of copies of a polynucleotide template sequence with the use of a polymerase. Polynucleotide synthesis (e.g., amplification) results in the incorporation of nucleotides into a polynucleotide (i.e., a primer), thereby forming a new polynucleotide molecule complementary to the polynucleotide template. The formed polynucleotide molecule and its template can be used as templates to synthesize additional polynucleotide molecules.

"DNA synthesis", according to the invention, includes, but is not limited to, PCR, the labelling of polynucleotide (i.e., for probes and oligonucleotide primers), polynucleotide sequencing.

As used herein, "polymerase" refers to an enzyme that catalyzes the polymerization of nucleotide (i.e., the polymerase activity). Generally, the enzyme will initiate synthesis at the 3'-end of the primer annealed to a polynucleotide template sequence, and will proceed toward the 5' end of the template strand. "DNA polymerase" catalyzes the polymerization of deoxynucleotides. In a preferred embodiment, the "DNA polymerase" of the invention is an Archaeal DNA polymerase. A "DNA polymerase" useful according to the invention includes,

but is not limited to those included in the section of the present specification entitled "Polymerases".

As used herein, "polypeptide that increases processivity and/or efficiency" refers to a domain that is a protein or a region of a protein or a protein complex, comprising a polypeptide sequence, or a plurality of peptide sequences wherein that region increases processivity, as defined herein, or increases salt resistance, as defined herein. A "polypeptide that increases processivity and/or efficiency useful according to the invention includes but is not limited to any of the domains included in Pavlov et al., supra or WO 01/92501, for example Sso7d, Sac7d, HMF-like proteins, PCNA homologs, and helix-hairpin-helix domains, for example derived from Topoisomerase V.

As used herein, "processivity" refers to the ability of a polynucleotide modifying enzyme, for example a polymerase, to remain attached to the template or substrate and perform multiple modification reactions. "Modification reactions" include but are not limited to polymerization, and exonucleolytic cleavage. "Processivity" also refers to the ability of a polynucleotide modifying enzyme, for example a polymerase, to modify relatively long (for example 0.5-1kb, 1-5kb or 5kb or more) tracts of nucleotides. "Processivity" also refers to the ability of a polynucleotide modifying enzyme, for example a DNA polymerase, to perform a sequence of polymerization steps without intervening dissociation of the enzyme from the growing DNA chains. "Processivity" can depend on the nature of the polymerase, the sequence of a DNA template, and reaction conditions, for example, salt concentration, temperature or the presence of specific proteins.

As used herein, "increased processivity" refers to an increase of 5-10%, preferably 10-50%, more preferably 50-100% or more, as compared to a wild type or mutant ARCHAEAL DNA polymerase that lacks a polypeptide that increases processivity as defined herein. Methods for measuring processivity of a DNA polymerase are generally known in the art, e.g., as described in Sambrook et al. 1989, In Molecular Cloning, 2nd Edition, CSH Press, 7.79-7.83 and 13.8, and as described in U.S. Patent Application with Serial No. 2002/0119467, hereby incorporated by reference. Processivity and increased processivity can be measured according the methods defined herein and in Pavlov et al., supra and WO

01/92501 A1. Processivity can also be measured by any known method in the art, e.g., as described in U.S. Patent No. 5,972,603, the entirety of which is incorporated herein by reference.

As used herein, the term "efficiency" of a DNA polymerase refers to a rate at which the DNA polymerase incorporates a nucleotide into a polynucleotide, or it may be defined as $N=No(1+E)^{CT}$ as described in "Amplification efficiency of thermostable DNA polymerases" Anal/ Biochem. 321 (2003) 226-235 (incorporated herein by reference). Methods for measuring the rate of incorporation are described herein below and are generally known in the art, e.g., as described in Leung et al. (1989) Technique 1:11-15 and Caldwell et al. (1992) PCR Methods Applic. 2:28-33, hereby incorporated by reference.

The term "efficiency" may be also defined in terns of $N=No(1+E)^{CT}$. Methods for calculating efficiency this way are known in the art, e.g., as described in Arezi et al., 2003 Analytical Biochem. 321:226/235, hereby incorporated by reference. Theoretically, the amount of product doubles during each PCR cycle; in other words, $N=No2^n$, where N is the number of amplified molecules, No is the initial number of molecules, and n is the number of amplification cycles. Experimentally, amplification efficiency (E) is less than perfect, ranging from 0 to 1, and therefore the real PCR equation is $N=No(1+E)^n$. At threshold cycle, where the emission intensity of the amplification product measured by a real-time PCR instrument (such as the Mx4000 Multiplex Quantitative PCR System; Stratagene, La Jolla, CA) is recorded as statistically significant above the background noise, the PCR equation transforms into $N=No(1+E)^{CT}$. This equation can also be written as $\log N=\log No + C_T \log(1+E)$, and therefore C_T is proportional to the negative of the log of the initial target copy number. thus, the plot of C_T versus the log of initial target copy number is a straight line, with a slope of $-[1/\log(1+E)]$ corresponding to amplification efficiency via the equation $E=10^{[-1/\text{slope}]}-1$.

As used herein, "increased efficiency" refers to an increase of 5-10%, preferably 10-50%, more preferably 50-100% or more, as compared to a wild type archaeal DNA polymerase.

As used herein, "increased salt resistance" refers to a polymerase that exhibits >50% activity at a salt concentration that is know to be greater than the maximum salt concentration at which the wild-type polymerase is active. The maximum salt concentration differs for each polymerase and is known in the art, or can be experimentally determined according to methods in the art. For example, Pfu is inhibited at 30mM (in PCR) so a Pfu enzyme with increased salt resistance would have significant activity (>50%) at salt concentrations above 30mM. A polymerase with increased salt resistance that is a chimera comprising a polypeptide that increases salt resistance, as defined herein, is described in Pavlov et al. supra and WO 01/92501 A1.

As used herein, a DNA polymerase with a "reduced DNA polymerization activity" is a DNA polymerase mutant comprising a DNA polymerization activity which is lower than that of the wild-type enzyme, e.g., comprising less than 10% DNA (e.g., less than 8%, 6%, 4%, 2% or less than 1%) polymerization activity of that of the wild-type enzyme. Methods used to generate characterize Pfu DNA polymerases with reduced DNA polymerization activity are disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated in their entirety.

As used herein, "thermostable" refers to an enzyme which is stable and active at temperatures as great as preferably between about 90-100^{0}C and more preferably between about 70-980C to heat as compared, for example, to a non-thermostable form of an enzyme with a similar activity. For example, a thermostable polynucleotide polymerase derived from thermophilic organisms such as *P. furiosus*, *M. jannaschii*, *A. fulgidus* or *P. horikoshii* are more stable and active at elevated temperatures as compared to a polynucleotide polymerase from *E. coli*. A representative thermostable polynucleotide polymerase isolated from *P. furiosus* (Pfu) is described in Lundberg et al., 1991, Gene, 108:1-6. Additional representative temperature stable polymerases include, e.g., polymerases extracted from the thermophilic bacteria *Thermus flavus, Thermus ruber, Thermus thermophilus, Bacillus stearothermophilus*

(which has a somewhat lower temperature optimum than the others listed), *Thermus lacteus, Thermus rubens, Thermotoga maritima,* or from thermophilic archaea *Thermococcus litoralis*, and *Methanothermus fervidus*.

Temperature stable polymerases are preferred in a thermocycling process wherein double stranded polynucleotides are denatured by exposure to a high temperature (about 95^0 C) during the PCR cycle.

As used herein, the term "template DNA molecule" refers to that strand of a polynucleotide from which a complementary polynucleotide strand is synthesized by a DNA polymerase, for example, in a primer extension reaction.

As used herein, the term "template dependent manner" is intended to refer to a process that involves the template dependent extension of a primer molecule (e.g., DNA synthesis by DNA polymerase). The term "template dependent manner" refers to polynucleotide synthesis of RNA or DNA wherein the sequence of the newly synthesised strand of polynucleotide is dictated by the well-known rules of complementary base pairing (see, for example, Watson, J. D. et al., In: Molecular Biology of the Gene, 4th Ed., W. A. Benjamin, Inc., Menlo Park, Calif. (1987)).

As used herein, an "amplified product" refers to the double strand polynucleotide population at the end of a PCR amplification reaction. The amplified product contains the original polynucleotide template and polynucleotide synthesized by DNA polymerase using the polynucleotide template during the PCR reaction.

As used herein, the term "abundance of polynucleotide" refers to the amount of a particular target polynucleotide sequence present in an amplification reaction, either before (e.g., the amount of the template polynucleotide), during (e.g., as in real-time PCR), or after the amplification (e.g., the amount of amplified product). The amount is generally measured as a relative amount in terms of concentration or copy number of the target sequence relative to the amount of a standard of known concentration or copy number. Alternatively, the amount in one unknown sample is measured relative to the amount in another unknown sample. As used herein, abundance of a polynucleotide is measured on the basis of the

intensity of a detectable label, most often a fluorescent label. The methods of the invention permit one to extrapolate the relative amount of one or more target sequences in a polynucleotide sample from the amplification profile of that target sequence or sequences from that sample.

The term "fidelity" as used herein refers to the accuracy of DNA polymerization by template-dependent DNA polymerase. The fidelity of a DNA polymerase is measured by the error rate (the frequency of incorporating an inaccurate nucleotide, i.e., a nucleotide that is not incorporated at a template-dependent manner). The accuracy or fidelity of DNA polymerization is maintained by both the polymerase activity and the 3'-5' exonuclease activity of a DNA polymerase. The term "high fidelity" refers to an error rate of 5×10^{-6} per base pair or lower. The fidelity or error rate of a DNA polymerase may be measured using assays known to the art. For example, the error rates of DNA polymerase mutants can be tested using the *lac*I PCR fidelity assay described in Cline, J., Braman, J.C., and Hogrefe, H.H. (96) NAR 24:3546-3551. Briefly, a 1.9kb fragment encoding the *lacIOlacZα* target gene is amplified from pPRIAZ plasmid DNA using 2.5U DNA polymerase (i.e. amount of enzyme necessary to incorporate 25 nmoles of total dNTPs in 30 min. at 72°C) in the appropriate PCR buffer. The *lac*I-containing PCR products are then cloned into lambda GT10 arms, and the percentage of *lac*I mutants (MF, mutation frequency) is determined in a color screening assay, as described (Lundberg, K.S., Shoemaker, D.D., Adams, M.W.W., Short, J.M., Sorge, J.A., and Mathur, E.J. (1991) Gene 180:1-8). Error rates are expressed as mutation frequency per bp per duplication (MF/bp/d), where bp is the number of detectable sites in the *lacI* gene sequence (349) and d is the number of effective target doublings. For each DNA polymerase mutant, at least two independent PCR amplifications are performed.

As used herein, "polynucleotide template" or "target polynucleotide template" or "template" refers to a polynucleotide containing an amplified region. The "amplified region," as used herein, is a region of a polynucleotide that is to be either synthesized by polymerase chain reaction (PCR). For example, an amplified region of a polynucleotide template resides between two sequences to which two PCR primers are complementary to.

As used herein, the term "primer" refers to a single stranded DNA or RNA molecule that can hybridize to a polynucleotide template and prime enzymatic synthesis of a second polynucleotide strand. A primer useful according to the invention is between 10 to 100 nucleotides in length, preferably 17-50 nucleotides in length and more preferably 17-45 nucleotides in length.

"Complementary" refers to the broad concept of sequence complementarity between regions of two polynucleotide strands or between two nucleotides through base-pairing. It is known that an adenine nucleotide is capable of forming specific hydrogen bonds ("base pairing") with a nucleotide which is thymine or uracil. Similarly, it is known that a cytosine nucleotide is capable of base pairing with a guanine nucleotide.

As used herein, the term "homology" refers to the optimal alignment of sequences (either nucleotides or amino acids), which may be conducted by computerized implementations of algorithms. "Homology", with regard to polynucleotides, for example, may be determined by analysis with BLASTN version 2.0 using the default parameters. "Homology", with respect to polypeptides (i.e., amino acids), may be determined using a program, such as BLASTP version 2.2.2 with the default parameters, which aligns the polypeptides or fragments being compared and determines the extent of amino acid identity or similarity between them. It will be appreciated that amino acid "homology" includes conservative substitutions, i.e. those that substitute a given amino acid in a polypeptide by another amino acid of similar characteristics. Typically seen as conservative substitutions are the following replacements: replacements of an aliphatic amino acid such as Ala, Val, Leu and Ile with another aliphatic amino acid; replacement of a Ser with a Thr or vice versa; replacement of an acidic residue such as Asp or Glu with another acidic residue; replacement of a residue bearing an amide group, such as Asn or Gln, with another residue bearing an amide group; exchange of a basic residue such as Lys or Arg with another basic residue; and replacement of an aromatic residue such as Phe or Tyr with another aromatic residue.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays altered

characteristics when compared to the wild-type gene or gene product. For example, a mutant DNA polymerase in the present invention is a DNA polymerase which exhibits a reduced uracil detection activity.

As used herein, "additive" refers to a reagent which can increase the processivity, efficiency, or heat or salt stability, including but not limited to, Pfu dUTPase (PEF), PCNA, RPA, ssb, antibodies, DMSO, betaine, 3'-5' exonuclease (e.g., Pfu G387P), Ncp7, recA, T4gp32.

As used herein "FEN-1 nuclease" refers to thermostable FEN-1 endonucleases useful according to the invention and include, but are not limited to, FEN-1 endonuclease purified from the "hyperthermophiles", e.g., from *M. jannaschii*, *P. furiosus* and *P. woesei*. See U.S. Patent No. 5,843,669, hereby incorporated by reference.

According to the methods of the present invention, the addition of FEN-1 in the amplification reaction dramatically increases the efficiency of PCR amplification. 400 ng to 4000 ng of FEN-1 may be used in each amplification reaction. Preferably 400-1000 ng, more preferably, 400-600 ng of FEN-1 is used in the amplification reaction. In a preferred embodiment of the invention, 400 ng FEN-1 is used.

As used herein, a "PCR enhancing factor" or a "Polymerase Enhancing Factor" (PEF) refers to a complex or protein possessing polynucleotide polymerase enhancing activity including, but not limited to, PCNA, RFC, helicases etc (Hogrefe et al., 1997, Strategies 10:93-96; and U.S. Patent No. 6,183,997, both of which are hereby incorporated by reference). '

Amino acid residues identified herein are preferred in the natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3557-3559, 1969, abbreviations for amino acid residues are as shown in the following Table I.

TABLE I

1-Letter	3-Letter	AMINO ACID
Y	Tyr	L-tyrosine

G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

Misincorporation, base deamination and other base modifications greatly increase as a consequence of PCR reaction conditions, for example, elevated temperature. This results in the progressive accumulation of base analogs (for example uracil or inosine) in the PCR reaction that ultimately inhibit Archaeal proofreading DNA polymerases, such as Pfu, Vent and Deep Vent DNA polymerases, severely limiting their processivity and/or efficiency.

The present invention provides a remedy to the above problem of PCR reactions by disclosing compositions for Archaeal DNA polymerase mutants which increase PCR

amplification processivity and/or efficiency and there uses thereof in PCR, including quantitative PCR and quantitative RT-PCR.

The mutant Archaeal DNA polymerases of the invention may provide for the use of fewer units of polymerase, may allow assays to be done using shorter extension times and/or may provide greater success in achieving higher yields and or longer products.

ARCHAEAL DNA POLYMERASES

There are 2 different classes of DNA polymerases which have been identified in archaea: 1. Family B/pol I type (homologs of Pfu from *Pyrococcus furiosus*) and 2. pol II type (homologs of *P. furiosus* DP1/DP2 2-subunit polymerase). DNA polymerases from both classes have been shown to naturally lack an associated 5' to 3' exonuclease activity and to possess 3' to 5' exonuclease (proofreading) activity. Suitable DNA polymerases (pol I or pol II) can be derived from archaea with optimal growth temperatures that are similar to the desired assay temperatures.

Thermostable Archaeal DNA polymerases include, but are mot limited to polymerases isolated from Pyrococcus species (furiosus, species GB-D, woesii, abysii, horikoshii), Thermococcus species (kodakaraensis KOD1, litoralis, species 9 degrees North-7, species JDF-3, gorgonarius), Pyrodictium occultum, and Archaeoglobus fulgidus. It is estimated that suitable archaea would exhibit maximal growth temperatures of >80-85^{0}C or optimal growth temperatures of >70-80^{0}C. Appropriate PCR enzymes from the Archaeal pol I DNA polymerase group are commercially available, including Pfu (Stratagene), KOD (Toyobo), Pfx (Life Technologies, Inc.), 9°N-7 (New England Biolabs, Inc), Vent (Tli) (New England BioLabs), Deep Vent (PGB-D) (New England BioLabs), Afu from *Archaeoglobus fulgidus* (e.g., Chalov et al., 2002, Dokl Biochem Biophys. 382:53-5), Mvo (Koniskyet al., 1994, J. Bacteriol. 176: 6402-6403), DTok (Bergseid, M., Scott, B. R., Mathur, S., Nielson, K. B., Shoemaker, D., Mathur, E. J. 1992, Strategies 5, 50), Pis (Kahler et al., 2000, J. Bacteriol. 182 655-663), Csy (Schleperet al., 1998, J. Bacteriol. 180 (19), 5003-5009), Sac (Datukishvili et al., 1996, Gene 177 (1-2), 271-273), Soh (Iwai et al., 2000, DNA Res. 7 (4), 243-251), Sso (Pisani et al., 1992, Nucleic Acids Res. 20 (11), 2711-2716), Poc (Uemori et al., 1995, J.

Bacteriol. 177 (8), 2164-2177), Ape (Kawarabayasi et al., 1999, DNA Res. 6 (2), 83-101), Tgo (Roche), and Pwo (Roche).

Additional Archaeal DNA polymerases related to those listed above are described in table 1 and in the following references: Archaea: A Laboratory Manual (Robb, F.T. and Place, A.R., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995 and *Thermophilic Bacteria* (Kristjansson, J.K.,ed.) CRC Press, Inc., Boca Raton, Florida, 1992.

The invention therefore provides for thermostable Archaeal DNA polymerases of either Family B/pol I type or pol II type with a reduced base analog detection activity.

Table II. Protein sequences for Archaeal DNA polymerases as represented by their accession numbers. Polynucleotide coding sequences can be found in references or nucleotide accession numbers identified in the Genbank database through the protein sequence accession numbers.

Vent Thermococcus litoralis

ACCESSION AAA72101

PID g348689

VERSION AAA72101.1 GI:348689

DBSOURCE locus THCVDPE accession M74198.1

THEST THERMOCOCCUS SP. (STRAIN TY)

ACCESSION O33845

PID g3913524

VERSION O33845 GI:3913524

DBSOURCE swissprot: locus DPOL_THEST, accession O33845

Pab Pyrococcus abyssi

ACCESSION P77916

PID g3913529

VERSION P77916 GI:3913529

DBSOURCE swissprot: locus DPOL_PYRAB, accession P77916

PYRHO Pyrococcus horikoshii

ACCESSION O59610

PID g3913526

VERSION O59610 GI:3913526

DBSOURCE swissprot: locus DPOL_PYRHO, accession O59610

PYRSE PYROCOCCUS SP. (STRAIN GE23)

ACCESSION P77932

PID g3913530

VERSION P77932 GI:3913530

DBSOURCE swissprot: locus DPOL_PYRSE, accession P77932

DeepVent Pyrococcus sp.

ACCESSION AAA67131

PID g436495

VERSION AAA67131.1 GI:436495

DBSOURCE locus PSU00707 accession U00707.1

Pfu Pyrococcus furiosus

ACCESSION P80061

PID g399403

VERSION P80061 GI:399403

DBSOURCE swissprot: locus DPOL_PYRFU, accession P80061

JDF-3 Thermococcus sp.

Unpublished

Baross gi|2097756|pat|US|5602011|12 Sequence 12 from patent US 5602011

9degN THERMOCOCCUS SP. (STRAIN 9°N-7).

ACCESSION Q56366

PID g3913540

VERSION Q56366 GI:3913540

DBSOURCE swissprot: locus DPOL_THES9, accession Q56366

KOD Pyrococcus sp.

ACCESSION BAA06142

PID g1620911

VERSION BAA06142.1 GI:1620911

DBSOURCE locus PYWKODPOL accession D29671.1

Tgo Thermococcus gorgonarius.

ACCESSION 4699806

PID g4699806

VERSION GI:4699806

DBSOURCE pdb: chain 65, release Feb 23, 1999

THEFM Thermococcus fumicolans

ACCESSION P74918

PID g3913528

VERSION P74918 GI:3913528

DBSOURCE swissprot: locus DPOL_THEFM, accession P74918

METTH Methanobacterium thermoautotrophicum

ACCESSION O27276

PID g3913522

VERSION O27276 GI:3913522

DBSOURCE swissprot: locus DPOL_METTH, accession O27276

Metja Methanococcus jannaschii

ACCESSION Q58295

PID g3915679

VERSION Q58295 GI:3915679

DBSOURCE swissprot: locus DPOL_METJA, accession Q58295

POC Pyrodictium occultum

ACCESSION B56277

PID g1363344

VERSION B56277 GI:1363344

DBSOURCE pir: locus B56277

ApeI Aeropyrum pernix

ACCESSION BAA81109

PID g5105797

VERSION BAA81109.1 GI:5105797

DBSOURCE locus AP000063 accession AP000063.1

ARCFU Archaeoglobus fulgidus

ACCESSION O29753

PID g3122019

VERSION O29753 GI:3122019

DBSOURCE swissprot: locus DPOL_ARCFU, accession O29753

Desulfurococcus sp. Tok.

ACCESSION 6435708

PID g64357089

VERSION GT:6435708

9oN-7

ACCESSION Q56366

VERSION Q56366 GI:3913540

Afu

ACCESSION O29753

VERSION O29753 GI:3122019

Mvo

ACCESSION P52025

VERSION P52025 GI:1706513

ACCESSION AAF27815

VERSION AAF27815.1 GI:6752664

Csy

ACCESSION AAC62712

VERSION AAC62712.1 GI:3599407

Sac

ACCESSION P95690

VERSION P95690 GI:3913538

Soh

ACCESSION BAA23994

VERSION BAA23994.1 GI:2696625

Sso

ACCESSION P26811

VERSION P26811 GI:12643274

Mutant DNA Polymerases

3'-5' exonuclease deficient

In one embodiment, the mutant DNA polymerase is a mutant with deficient 3'-5' exonuclease activity.

DNA polymerases lacking 3'-5' exonuclease (proofreading) activity are preferred for applications requiring nucleotide analog incorporation (e.g., DNA sequencing) to prevent removal of nucleotide analogs after incorporation. The 3'-5' exonuclease activity associated with proofreading DNA polymerases can be reduced or abolished by mutagenesis. Sequence comparisons have identified three conserved motifs (exo I (DXE), II (NX_{2-3}(F/Y)D), III (YX_3D)) in the 3'-5' exonuclease domain of DNA polymerases (reviewed V. Derbyshire, J.K. Pinsonneault, and C.M. Joyce, Methods Enzymol. 262, 363 (1995)). For example, replacement of any of the conserved aspartic or glutamic acid residues with alanine has been shown to abolish the exonuclease activity of numerous DNA polymerases, including Archaeal DNA polymerases such as Vent (H. Kong, R.B. Kucera, and W.E. Jack, J. Biol. Chem. 268, 1965 (1993)) and Pfu (Stratagene, unpublished). It is understood, according to the present invention, that other amino acids within or outside the exonuclease motifs may also be mutated to render the DNA polymerase deficient in 3'-5' exonuclease activity (e.g., by affecting the tertiary structure of the exonuclease domain). Conservative substitutions lead to reduced exonuclease activity, as shown for mutants of the Archaeal 9° N-7 DNA polymerase (M.W. Southworth, H. Kong, R.B. Kucera, J. Ware, H. Jannasch, and F.B. Perler, Proc. Natl. Acad. Sci. 93, 5281 (1996)).

In one embodiment, a 3'-5' exonuclease deficient JDF-3, KOD, or Pfu DNA polymerase is produced.

In one embodiment of the invention, the mutant DNA polymerase contains a mutation at a position corresponding to D141 and/or E143 of JDF-3 DNA polymerase.

JDF-3 DNA polymerase mutants exhibiting substantially reduced 3'-5' exonuclease activity (e.g., with one or more mutations as D141A, D141N, D141S, D141T, D141E and E143A) were prepared by introducing amino acid substitutions at the conserved 141D or 143E residues in the exo I domain, as described in U.S. Patent Application with Serial No. 10/223,650, hereby incorporated by reference.

It is appreciated that one skilled in the art would be able to make an Archaeal DNA polymerase with deficient 3'-5' exonuclease activity by comparing the sequence of the Archaeal DNA polymerase with the sequence of JDF-3 DNA polymerase and by mutating the amino acids within the corresponding conserved exo I, II, or III motifs. In addition, it is also appreciated that one skilled in the art would be able to make an Archaeal DNA polymerase with deficient 3'-5' exonuclease activity by mutating one or more amino acid within the corresponding exo I, II, and III motifs.

Assays for DNA polymerase activity and 3'-5' exonuclease activity can be found in DNA Replication 2nd Ed., Kornberg and Baker, supra; Enzymes, Dixon and Webb, Academic Press, San Diego, Calif. (1979), as well as other publications available to the person of ordinary skill in the art.

Suitable exonuclease activity assays include one described in Hogrefe et al (Hogrefe et al., 2001, Methods in Enzymology, 343:91-116, incorporated by reference). Another assay employs double-stranded λ DNA, which has been uniformly labeled with ^{3}H S-adenosyl methionine (NEN #NET-155) and *Sss* I methylase (NEB), and then restriction digested with *Pal* I (Kong et al., 1993, J. Biol. Chem. 268:1965). Using double-stranded labeled DNA templates, one can determine specificity by measuring whether cpms decrease (3'-5' exonuclease) with the addition of dNTPs (10-100μM). A typical exonuclease reaction cocktail consists of 1x reaction buffer and 20μg/ml ^{3}H-labeled digested double-stranded λ DNA (~10^{6} cpms/ml), prepared as described (Kong et al., supra). Exonuclease activity can be measured in the appropriate PCR buffer or in a universal assay buffer such as 70mM Tris HCl (pH 8.8), 2mM $MgCl_2$, 0.1% Triton-X, and 100μg/ml BSA.

Percent exonuclease activity can be determined as: (corrected cpms for mutants)/(corrected cpms for wt DNA polymerase). To more precisely quantify % activity, cpms released can be converted into units of exonuclease activity. One unit of exonuclease activity is defined as the amount of enzyme that catalyzes the acid-solubilization of 10nmoles of total dNMPs in 30 minutes at a defined temperature. To determine units, background (average "minimum cpms" value) is first subtracted from the average sample cpms. Nmoles dNMPs released is calculated using the following equation:

$$\frac{\text{(corrected sample cpms)}}{\text{total cpms}} \times \frac{\text{(ng DNA)}}{\text{reaction}} \times \frac{\text{(1nmole dNMP)}}{\text{(330ng dNMP)}}$$

Units of exonuclease activity (in 30 minutes) can then be determined as:

$$\frac{\text{(nmoles dNMPs released per hr)}}{2} \times \frac{\text{(1 unit)}}{\text{(10nmoles dNMPs released)}}$$

Exonuclease specific activity (U/mg) can be extrapolated from the slope of the linear portion of units versus enzyme amount plots. Finally, % activity can be determined as:

$$\frac{\text{specific exonuclease activity (U/mg) of mutant DNA polymerase}}{\text{specific exonuclease activity (U/mg) of wt DNA polymerase}}$$

In addition to the substrate described above, exonuclease activity can be also be quantified using [^{3}H]-*E. coli* genomic DNA (NEN #NET561; 5.8μCi/μg), a commercially-available substrate. A typical exonuclease reaction cocktail consists of 25ng/ml ^{3}H-labeled *E. coli* genomic DNA and 975 ng/ml cold E. coli genomic DNA in 1x reaction buffer. Assays are performed as described above.

Reduced uracil base detection

In one embodiment of the invention, the Archaeal polymerase is a mutant polymerase having reduced uracil base detection.

Examination of Archaeal DNA polymerases revealed the presence of a distinct "pocket" located on a surface-exposed face toward the outer edge of the polymerases (Fogg, et al., 2002, Nature structural Biology, 9:922-927, hereby incorporated by reference in its entirety). The pocket is formed entirely by residues from four conserved segments in the Archaeal DNA polymerase sequences. Corresponding to Pfu DNA polymerase sequence, the base of the pocket is formed by the main chain and side chains of amino acids Pro36, Tyr 37, and Ile 38, one face of the pocket is formed by amino acids 90-97, another face is formed by residues 111-116, and by Pro 115.

An wild type Archaeal DNA polymerase or an Archaeal DNA polymerase with deficient 3'-5' exonuclease activity may be mutated at or more amino acid positions corresponding to Pro36, Tyr 37, Ile 38, amino acids 90-97, residues 111-116, and Pro 115 in wild type Pfu DNA polymerase, e.g., as described in U.S Patent Application with Serial Number 10/408,601, filed April 7, 2003, hereby incorporated by reference in its entirety.

In one embodiment of the invention, the mutant DNA polymerase is encoded by a polynucleotide sequence selected from SEQ ID Nos 17-24, wherein the codon encoding amino acid residue Valine at position 93 is replaced by the one of the following codons:

Codons encoding Arginine: AGA, AGG, CGA, CGC, CGG, CGT

Codons encoding Glutamic acid: GAA, GAG

Codons encoding Aspartic acid: GAT, GAC

Codons encoding Lysine: AAA, AAG

Codons encoding Glutamine: CAA, CAG

Codons encoding Asparagine AAC, AAU

In one embodiment, a mutant DNA polymerase has an amino acid sequence selected from the sequences of SEQ ID NOS: 27-34, wherein Valine at position 93 is replaced by one of Arginine, Glutamic acid, Aspartic acid, Lysine, Glutamine, and Asparagine.

Alternatively, the mutant DNA polymerase may be a Pfu DNA polymerase having a deletion of Valine at position 93 as shown in SEQ ID NO: 35, or alternatively, having a deletion of Aspartic acid at position 92, Valine at position 93, and Proline at position 94 as shown in SEQ ID NO: 36. Similarly, the mutant DNA polymerase may be a Pfu DNA polymerase having a deletion of the codon GTT encoding Valine at position 93 as shown in SEQ ID NO: 25, or alternatively having a deletion of the successive codons GAT, GTT, and CCC which encode residues Aspartic acid, Valine, and Proline at positions 92, 93, and 94 respectively as shown in SEQ ID NO: 26.

In one embodiment, a Pfu, KOD or JDF-3 DNA polymerase mutants exhibiting substantially reduced 3'-5' exonuclease activity (e.g., with one or more mutations as D141A, D141N, D141S, D141T, D141E and E143A) are mutated to further comprise one or more mutations at corresponding positions to Pro36, Tyr 37, Ile 38, amino acids 90-97, residues 111-116, and Pro 115 of wild type Pfu DNA polymerase.

The present invention encompass making an Archaeal DNA polymerase with reduced uracil base detection by comparing the sequence of the Archaeal DNA polymerase with the sequence of Pfu DNA polymerase and by mutating the amino acids within the corresponding conserved residues within the pocket forming amino acids. In addition, one skilled in the art

would be able to make an Archaeal DNA polymerase with reduced uracil base detection by mutating one or more amino acid within these amino acid positions.

Increased reverse transcriptase activity

Amino acid changes at the position corresponding to L408 of JDF-3 Family B DNA polymerase which lead to increased reverse transcriptase activity tend to introduce cyclic side chains, such as phenylalanine, tryptophan, histidine or tyrosine as described in U.S. Patent Application with Serial No. 10/435,766, hereby incorporated by reference. While the amino acids with cyclic side chains are demonstrated herein to increase the reverse transcriptase activity of Archaeal Family B DNA polymerases, other amino acid changes at the LYP motif are contemplated to have effects on the reverse transcriptase activity. Thus, in order to modify the reverse transcriptase activity of another Archaeal Family B DNA polymerase, one would first look to modify the LYP motif of Region II, particularly the L or other corresponding amino acid of the LYP motif, first substituting cyclic side chains and assessing reverse transcriptase activity relative to wild-type as disclosed herein below in "Methods of Evaluating Mutants for Increased RT Activity." If necessary or if desired, one can subsequently modify the same position in the LYP motif with additional amino acids and similarly assess the effect on activity. Alternatively, or in addition, one can modify the other positions in the LYP motif and similarly assess the reverse transcriptase activity.

Methods for assaying reverse transcriptase (RT) activity based on the RNA-dependent synthesis of DNA have been well known in the art, e.g., as described in U.S. Patent No. 3,755,086; Poiesz et al., (1980) Proc. Natl. Acad. Sci. USA, 77: 1415; Hoffman et al., (1985) Virology 147: 326; all hereby incorporated by reference.

Recently, highly sensitive PCR based assays have been developed that can detect RNA-dependent DNA polymerase in the equivalent of one to ten particles (Silver et al. (1993) Nucleic Acids Res. 21: 3593-4; U.S. Patent No. 5,807,669). One such assay, designated as PBRT (PCR-based reverse transcriptase), has been used to detect RT activity in a variety of samples (Pyra et al. (1994) Proc. Natl. Acad. Sci. USA 51: 1544-8; Boni, et al. (1996) J. Med. Virol. 49: 23-28). This assay is 10^6 -10^7 more sensitive than the conventional RT assay.

Other useful RT assays include, but are not limited to, one-step fluorescent probe product-enhanced reverse transcriptase assay described in Hepler, R. W., and Keller, P. M. (1998). Biotechniques 25(1), 98-106; an improved product enhanced reverse transcriptase assay described in Chang, A., Ostrove, J. M., and Bird, R. E. (1997) J Virol Methods 65(1), 45-54; an improved non-radioisotopic reverse transcriptase assay described in Nakano et al., (1994) Kansenshogaku Zasshi 68(7), 923-3 1; a highly sensitive qualitative and quantitative detection of reverse transcriptase activity as described in Yamamoto, S., Folks, T. M., and Heneine, W. (1996) J Virol Methods 61(1-2), 135-43, all references hereby incorporated by reference.

RT activity can be measured using radioactive or non-radioactive labels.

In one embodiment, 1 μl of appropriately purified DNA polymerase mutant or diluted bacterial extract (i.e., heat-treated and clarified extract of bacterial cells expressing a cloned polymerase or mutated cloned polymerase) is added to 10 μl of each nucleotide cocktail (200μM dATP, 200μM dGTP, 200μM dCTP and 5 μCi/ml α-^{33}P dCTP and 200μM dTTP, a RNA template, 1X appropriate buffer, followed by incubation at the optimal temperature for 30 minutes (e.g., 72°C for Pfu DNA polymerase), for example, as described in Hogrefe et al., 2001, Methods in Enzymology, 343:91-116. Extension reactions are then quenched on ice, and 5μl aliquots are spotted immediately onto DE81 ion-exchange filters (2.3cm; Whatman #3658323). Unincorporated label is removed by 6 washes with 2 x SCC (0.3M NaCl, 30mM sodium citrate, pH 7.0), followed by a brief wash with 100% ethanol. Incorporated radioactivity is then measured by scintillation counting. Reactions that lack enzyme are also set up along with sample incubations to determine "total cpms" (omit filter wash steps) and "minimum cpms"(wash filters as above). Cpms bound is proportional to the amount of RT activity present per volume of bacterial extract or purified DNA polymerase.

In another embodiment, the RT activity is measured by incorporation of non-radioactive digoxigenin labeled dUTP into the synthesized DNA and detection and quantification of the incorporated label essentially according to the method described in Holtke, H.-J.; Sagner, G; Kessler, C. and Schmitz, G. (1992) Biotechniques 12, 104-113. The reaction is performed in a reaction mixture consists of the following components: 1 μg of

polydA-$(dT)_{15}$, 33 µM of dTTP, 0.36 µM of labeled-dUTP, 200 mg/ml BSA, 10 mM Tris-HCl, pH 8.5, 20 mM KCl, 5 mM $MgCl_2$, 10 mM DTE and various amounts of DNA polymerase. The samples are incubated for 30 min. at 50 °C, the reaction is stopped by addition of 2 µ0.5 M EDTA, and the tubes placed on ice. After addition of 8 µl 5 M NaCl and 150 µl of Ethanol (precooled to –20°C) the DNA is precipitated by incubation for 15 min on ice and pelleted by centrifugation for 10 min at 13000xrpm and 4°C. The pellet is washed with 100 µl of 70% Ethanol (precooled to –20°C) and 0.2 M NaCl, centrifuged again and dried under vacuum.

The pellets are dissolved in 50 µl Tris-EDTA (10 mM/0.1 mM; pH 7.5). 5 µl of the sample are spotted into a well of a nylon membrane bottomed white microwave plate (Pall Filtrationstechnik GmbH, Dreieich, FRG, product no: SM045BWP). The DNA is fixed to the membrane by baking for 10 min. at 70°C. The DNA loaded wells are filled with 100 µl of 0.45 µm-filtrated 1% blocking solution (100 mM maleic acid, 150 mM NaCl, 1% (w/v) casein, pH 7.5). All following incubation steps are done at room temperature. After incubation for 2 min. the solution is sucked through the membrane with a suitable vacuum manifold at -0.4 bar. After repeating the washing step, the wells are filled with 100 µl of a 1:10,000-dilution of Anti-digoxigenin-AP, Fab fragments (Boehringer Mannheim, FRG, no: 1093274) diluted in the above blocking solution. After incubation for 2 min. and sucking this step is repeated once. The wells are washed twice under vacuum with 200 µl each time washing-buffer 1 (100 mM maleic-acid, 150 mM NaCl, 0.3%(v/v) Tween.TM. 20, pH 7.5). After washing another two times under vacuum with 200 µl each time washing-buffer 2 (10 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) the wells are incubated for 5 min with 50 µl of CSPD™ (Boehringer Mannheim, no: 1655884), diluted 1:100 in washing-buffer 2, which serves as a chemiluminescent substrate for the alkaline phosphatase. The solution is sucked through the membrane and after 10 min incubation the RLU/s (Relative Light Unit per second) are detected in a Luminometer e.g. MicroLumat LB 96 P (EG&G Berthold, Wilbad, FRG). With a serial dilution of Taq DNA polymerase a reference curve is prepared from which the linear range serves as a standard for the activity determination of the DNA polymerase to be analyzed.

U.S. Patent 6,100,039 (incorporated hereby by reference) describes another useful process for detecting reverse transcriptase activity using fluorescence polarization: the reverse transcriptase activity detection assays are performed using a Beacon™ 2000 Analyzer. The following reagents are purchased from commercial sources: fluorescein-labeled oligo dA-F (Bio.Synthesis Corp., Lewisville, Tex.), AMV Reverse Transcriptase (Promega Corp., Madison, Wis.), and Polyadenylic Acid Poly A (Pharmacia Biotech, Milwaukee, Wis.). The assay requires a reverse trancriptase reaction step followed by a fluorescence polarization-based detection step. The reverse transcriptase reactions are completed using the directions accompanying the kit. In the reaction 20 ng of Oligo (dT) were annealed to 1 μg of Poly A at 70°C for 5 minutes. The annealed reactions are added to an RT mix containing RT buffer and dTTP nucleotides with varying units of reverse transcriptase (30, 15, 7.5, 3.8, and 1.9 Units/Rxn). Reactions are incubated at 37 °C in a water bath. 5 μl aliquots are quenched at 5, 10, 15, 20, 25, 30, 45, and 60 minutes by adding the aliquots to a tube containing 20 μl of 125 mM NaOH. For the detection step, a 75 μl aliquot of oligo dA-F in 0.5 M Tris, pH 7.5, is added to each quenched reaction. The samples are incubated for 10 minutes at room temperature. Fluorescence polarization in each sample was measured using the Beacon™ 2000 Analyzer.

Additional Mutations

The mutant DNA polymerase of the present invention may contain additional mutations.

In one embodiment, the mutant DNA polymerase of the present invention contains a mutation which reduces its analog discrimination activity as described in U.S. Application with Serial Number 10/223,650, hereby incorporated by reference in its entirety.

In another embodiment, the mutant DNA polymerase of the present invention contains a mutation which reduces its polymerization activity as described in U.S. Patent Application with Serial No. Serial No.: 10/227,110, hereby incorporated by reference.

In another embodiment, the mutant DNA polymerase of the present invention is a chimeric protein, e.g., as described in U.S. Patent Application with Serial No. 10/324,846, hereby incorporated by reference in its entirety.

In another embodiment, the mutant DNA polymerase of the present invention also contains a mutation which increases the RT activity.

II. PREPARING MUTANT DNA POLYMERASE

Cloned wild-type DNA polymerases may be modified to generate forms exhibiting deficient 3'-5' exonuclease and/or reduced base analog detection activity (as well as other modified activities) by a number of methods. These include the methods described below and other methods known in the art. Any proofreading Archaeal DNA polymerase can be used to prepare for DNA polymerase with reduced base analog detection activity in the invention.

GENETIC MODIFICATIONS - MUTAGENESIS

Direct comparison of DNA polymerases from diverse organisms indicates that the domain structure of these enzymes is highly conserved and in many instances, it is possible to assign a particular function to a well-defined domain of the enzyme. The conserved exo motifs and the uracil pocket among the Archaeal DNA polymerases provide a useful model to direct genetic modifications for preparing DNA polymerase with desired activity.

The preferred method of preparing a DNA polymerase with desired activity, e.g., deficient 3'-5' exo activity and/or reduced base analog detection activity is by genetic modification (e.g., by modifying the DNA sequence of a wild-type DNA polymerase, or a mutant DNA polymerase). A number of methods are known in the art that permit the random as well as targeted mutation of DNA sequences (see for example, Ausubel et. al. Short Protocols in Molecular Biology (1995) 3rd Ed. John Wiley & Sons, Inc.). In addition, there are a number of commercially available kits for site-directed mutagenesis, including both conventional and PCR-based methods. Examples include the EXSITE™ PCR-Based Site-directed Mutagenesis Kit available from Stratagene (Catalog No. 200502) and the QUIKCHANGE™ Site-directed mutagenesis Kit from Stratagene (Catalog No. 200518), and

the CHAMELEON® double-stranded Site-directed mutagenesis kit, also from Stratagene (Catalog No. 200509).

In addition DNA polymerases with deficient 3'-5' exo activity and/or reduced base analog detection activity may be generated by insertional mutation or truncation (N-terminal, internal or C-terminal) according to methodology known to a person skilled in the art.

Older methods of site-directed mutagenesis known in the art rely on sub-cloning of the sequence to be mutated into a vector, such as an M13 bacteriophage vector, that allows the isolation of single-stranded DNA template. In these methods, one anneals a mutagenic primer (i.e., a primer capable of annealing to the site to be mutated but bearing one or mismatched nucleotides at the site to be mutated) to the single-stranded template and then polymerizes the complement of the template starting from the 3' end of the mutagenic primer. The resulting duplexes are then transformed into host bacteria and plaques are screened for the desired mutation.

More recently, site-directed mutagenesis has employed PCR methodologies, which have the advantage of not requiring a single-stranded template. In addition, methods have been developed that do not require sub-cloning. Several issues must be considered when PCR-based site-directed mutagenesis is performed. First, in these methods it is desirable to reduce the number of PCR cycles to prevent expansion of undesired mutations introduced by the polymerase. Second, a selection must be employed in order to reduce the number of non-mutated parental molecules persisting in the reaction. Third, an extended-length PCR method is preferred in order to allow the use of a single PCR primer set. And fourth, because of the non-template-dependent terminal extension activity of some thermostable polymerases it is often necessary to incorporate an end-polishing step into the procedure prior to blunt-end ligation of the PCR-generated mutant product.

The protocol described below accommodates these considerations through the following steps. First, the template concentration used is approximately 1000-fold higher than that used in conventional PCR reactions, allowing a reduction in the number of cycles from 25-30 down to 5-10 without dramatically reducing product yield. Second, the restriction endonuclease Dpn I (recognition target sequence: 5-Gm6ATC-3, where the A residue is

methylated) is used to select against parental DNA, since most common strains of E. coli Dam methylate their DNA at the sequence 5-GATC-3. Third, Taq Extender is used in the PCR mix in order to increase the proportion of long (i.e., full plasmid length) PCR products. Finally, Pfu DNA polymerase is used to polish the ends of the PCR product prior to intramolecular ligation using T4 DNA ligase.

A non-limiting example for the isolation of mutant Archaeal DNA polymerases exhibiting reduced uracil detection activity is described in detail as follows:

Plasmid template DNA (approximately 0.5 pmole) is added to a PCR cocktail containing: 1x mutagenesis buffer (20 mM Tris HCl, pH 7.5; 8 mM $MgCl_2$; 40 μg/ml BSA); 12-20 pmole of each primer (one of skill in the art may design a mutagenic primer as necessary, giving consideration to those factors such as base composition, primer length and intended buffer salt concentrations that affect the annealing characteristics of oligonucleotide primers; one primer must contain the desired mutation, and one (the same or the other) must contain a 5' phosphate to facilitate later ligation), 250 μM each dNTP, 2.5 U Taq DNA polymerase, and 2.5 U of Taq Extender (Available from Stratagene; See Nielson et al. (1994) Strategies 7: 27, and U.S. Patent No. 5,556,772). Primers can be prepared using the triester method of Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185-3191, incorporated herein by reference. Alternatively automated synthesis may be preferred, for example, on a Biosearch 8700 DNA Synthesizer using cyanoethyl phosphoramidite chemistry.

The PCR cycling is performed as follows: 1 cycle of 4 min at 94°C, 2 min at 50°C and 2 min at 72°C; followed by 5-10 cycles of 1 min at 94°C, 2 min at 54°C and 1 min at 72°C. The parental template DNA and the linear, PCR-generated DNA incorporating the mutagenic primer are treated with DpnI (10 U) and Pfu DNA polymerase (2.5U). This results in the DpnI digestion of the in vivo methylated parental template and hybrid DNA and the removal, by Pfu DNA polymerase, of the non-template-directed Taq DNA polymerase-extended base(s) on the linear PCR product. The reaction is incubated at 37°C for 30 min and then transferred to 72°C for an additional 30 min. Mutagenesis buffer (115 ul of 1x) containing 0.5 mM ATP is added to the DpnI-digested, Pfu DNA polymerase-polished PCR products. The solution is mixed and 10 ul are removed to a new microfuge tube and T4 DNA ligase (2-4 U)

is added. The ligation is incubated for greater than 60 min at 37°C. Finally, the treated solution is transformed into competent E. coli according to standard methods.

Methods of random mutagenesis, which will result in a panel of mutants bearing one or more randomly situated mutations, exist in the art. Such a panel of mutants may then be screened for those exhibiting reduced uracil detection activity relative to the wild-type polymerase (e.g., by measuring the incorporation of 10nmoles of dNTPs into polymeric form in 30 minutes in the presence of 200μM dUTP and at the optimal temperature for a given DNA polymerase). An example of a method for random mutagenesis is the so-called "error-prone PCR method". As the name implies, the method amplifies a given sequence under conditions in which the DNA polymerase does not support high fidelity incorporation. The conditions encouraging error-prone incorporation for different DNA polymerases vary, however one skilled in the art may determine such conditions for a given enzyme. A key variable for many DNA polymerases in the fidelity of amplification is, for example, the type and concentration of divalent metal ion in the buffer. The use of manganese ion and/or variation of the magnesium or manganese ion concentration may therefore be applied to influence the error rate of the polymerase.

Genes for desired mutant DNA polymerases generated by mutagenesis may be sequenced to identify the sites and number of mutations. For those mutants comprising more than one mutation, the effect of a given mutation may be evaluated by introduction of the identified mutation to the wild-type gene by site-directed mutagenesis in isolation from the other mutations borne by the particular mutant. Screening assays of the single mutant thus produced will then allow the determination of the effect of that mutation alone.

A person of average skill in the art having the benefit of this disclosure will recognize that polymerases with deficient 3'-5' exo activity and/or reduced uracil detection derived from JDF-3 or PFU or other exo^+ DNA polymerases including Vent DNA polymerase, JDF-3 DNA polymerase, Tgo DNA polymerase, and the like may be suitably used in the subject compositions.

In one embodiment, the invention provides DNA polymerase selected from Pfu, Tgo, JDF-3 and KOD comprising one or more mutations at V93, and which demonstrate reduced uracil detection activity.

In another embodiment, the invention provides DNA polymerase selected from Pfu, Tgo, JDF-3 and KOD comprising one or more mutations at D141 and/or E143, which is deficient in 3'-5' exonuclease activity.

In another embodiment, the invention provides DNA polymerase selected from Pfu, Tgo, JDF-3 and KOD comprising one or more mutations at V93, and which demonstrate reduced uracil detection activity, and further comprising one or more mutations at D141 and/or E143, which is deficient in 3'-5' exonuclease activity.

In another embodiment, the invention provides DNA polymerase selected from Pfu, Tgo, JDF-3 and KOD comprising one or more mutations at V93, and which demonstrate reduced uracil detection activity, and further comprising one or more mutations at D141 and/or E143, which is deficient in 3'-5' exonuclease activity, as well as a mutation at L408, which has an increased reverse transcriptase activity.

The enzyme of the subject composition may comprise DNA polymerases that have not yet been isolated.

In preferred embodiments of the invention, the mutant Pfu DNA polymerase harbors an amino acid substitution at amino acid position, V93. In a preferred embodiment, the mutant Pfu DNA polymerase of the invention contains a Valine to Arginine, Valine to Glutamic acid, Valine to Lysine, Valine to Aspartic Acid, or Valine to Asparagine substitution at amino acid position 93.

The invention further provides for mutant Archaeal DNA polymerases with reduced base analog detection activity that contains a Valine to Arginine, Valine to Glutamic acid, Valine to Lysine, Valine to Aspartic Acid, Valine to Glutamine, or Valine to Asparagine

substitution at amino acid position 93. In particular, Figure 6 shows mutant Archaeal DNA polymerases of the invention with reduced base analog detection activity.

According to the invention, V93 mutant Pfu DNA polymerases with reduced uracil detection activity may contain one or more additional mutations that reduce or abolish one or more additional activities of V93 Pfu DNA polymerases, e.g., DNA polymerization activity or 3'-5' exonuclease activity. In one embodiment, the V93 mutant Pfu DNA polymerase according to the invention contains one or more mutations that renders the DNA polymerase 3'-5' exonuclease deficient. In another embodiment, the V93 mutant Pfu DNA polymerase according to the invention contains one or more mutations that the DNA polymerization activity of the V93 Pfu DNA polymerase.

In another embodiment, a mutant Archaeal dna polymerase is a chimera that further comprises a polypeptide that increases processivity and/or increases salt resistance. A polypeptide useful according to the invention and methods of preparing chimeras are described in WO 01/92501 A1 and Pavlov et al., 2002, Proc. Natl. Acad. Sci USA, 99:13510-13515. Both references are herein incorporated in their entirety.

The invention provides for V93Rmutant Pfu DNA polymerases with reduced uracil detection activity containing one or mutations that reduce DNA polymerization as disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated in their entirety.

In a preferred embodiment, the invention provides for a V93R/ G387P, V93E/ G387P, V93D/G387P, V93K/G387P and V93N/G387P double mutant Pfu DNA polymerase with reduced DNA polymerization activity and reduced uracil detection activity.

The invention further provides for V93R, V93E, V93D, V93K and V93N mutant Pfu DNA polymerases with reduced uracil detection activity containing one or mutations that

reduce or eliminate 3'-5' exonuclease activity as disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000).

In a preferred embodiment, the invention provides for a V93R/D141A/E143A triple mutant Pfu DNA polymerase with reduced 3'-5' exonuclease activity and reduced uracil detection activity.

The invention further provides for combination of one or more mutations that may increase or eliminate base analog detection activity of an Archaeal DNA polymerase.

DNA polymerases containing additional mutations are generated by site directed mutagenesis using the Pfu DNA polymerase or Pfu V93R cDNA as a template DNA molecule, according to methods that are well known in the art and are described herein.

Methods used to generate Pfu DNA polymerases with reduced DNA polymerization activity are disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated in their entirety.

Methods for generating 3'-5' exonuclease deficient Pfu are disclosed in U.S. Patent No. 5,489,523, incorporated herein by reference.

Methods used to generate 3'-5' exonuclease deficient JDF-3 DNA polymerases including the D141A and E143A mutations are disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000). A person skilled in the art in possession of the V93 Pfu DNA polymerase cDNA and the teachings of the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000) would have no difficulty introducing both the corresponding D141A and E143A mutations or other 3'-5' exonuclease mutations into the V93 Pfu DNA polymerase cDNA, as disclosed in the pending U.S. patent application Serial No.: 09/698,341, using established site directed mutagenesis methodology.

Such methods (e.g., for Pfu and JDF-3) can be readily used to generate other 3'-5' exonuclease deficient archaeal DNA polymerase. Sequence alignment techniques are known in the art and are taught herein. One skilled in the art would appreciate the teaching of the present invention and can identify amino acid sequences to mutate by aligning Pfu or JDF-3 sequence with another archaeal DNA polymerase.

Methods of preparing chimeras according to the invention are described in WO 01/92501 A1 and Pavlov et al., 2002, Proc. Natl. Acad. Sci USA, 99:13510-13515. Both references are herein incorporated in their entirety.

In one embodiment, the Pfu mutants are expressed and purified as described in U.S. Patent No. 5,489,523, hereby incorporated by reference in its entirety.

III. METHODS OF EVALUATING MUTANTS FOR REDUCED BASE ANALOG DETECTION ACTIVITY AND 3'-5' EXONUCLEASE ACTIVITY, ETC.

Random or site-directed mutants generated as known in the art or as described herein and expressed in bacteria may be screened for reduced uracil detection activity by several different assays. Embodiments for the expression of mutant and wild type enzymes is described herein. In one method, exo^+ DNA polymerase proteins expressed in lytic lambda phage plaques generated by infection of host bacteria with expression vectors based on, for example, Lambda ZapII®, are transferred to a membrane support. The immobilized proteins are then assayed for polymerase activity on the membrane by immersing the membranes in a buffer containing a DNA template and the unconventional nucleotides to be monitored for incorporation.

Mutant polymerase libraries may be screened using a variation of the technique used by Sagner et al (Sagner, G., Ruger, R., and Kessler, C. (1991) Gene 97:119-123). For this approach, lambda phage clones are plated at a density of 10-20 plaques per square centimeter and replica plated. Proteins present in the plaques are transferred to filters and moistened with polymerase screening buffer (50mM Tris (pH 8.0), 7mM MgCl2, 3mM β-ME). The filters are kept between layers of plastic wrap and glass while the host cell proteins are heat-inactivated by incubation at 65°C for 30 minutes. The heat-treated filters are then transferred

to fresh plastic wrap and approximately 35µl of polymerase assay cocktail are added for every square centimeter of filter. The assay cocktail consists of 1X cloned Pfu (cPfu) magnesium free buffer (1X buffer is 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH4)_2SO_4$, 100 µg/ml bovine serum albumin (BSA), and 0.1% Triton X-100; Pfu Magnesium-free buffer may be obtained from Stratagene (Catalog No. 200534)), 125 ng/ml activated calf thymus or salmon sperm DNA, 200µM dATP, 200µM dGTP, 200µM dCTP and 5 µCi/ml α-^{33}P dCTP and 200µM dUTP or 200µM dTTP. The filters, in duplicate, are placed between plastic wrap and a glass plate and then incubated at 65°C for one hour, and then at 70°C for one hour and fifteen minutes. Filters are then washed three times in 2X SSC for five minutes per wash before rinsing twice in 100% ethanol and vacuum drying. Filters are then exposed to X-ray film (approximately 16 hours), and plaques that incorporate label in the presence of 200µM dUTP or 200µM dTTP are identified by aligning the filters with the original plate bearing the phage clones. Plaques identified in this way are re-plated at more dilute concentrations and assayed under similar conditions to allow the isolation of purified plaques.

In assays such as the one described above, the signal generated by the label is a direct measurement of the polymerization activity of the polymerase in the presence of 200 µM dUTP as compared to the polymerase activity of the same mutant polymerase in the presence of 200 µM dTTP. A plaque comprising a mutant DNA polymerase with reduced uracil detection activity as compared to that of the wild-type enzyme can then be identified and further tested in primer extension assays in which template dependent DNA synthesis is measured in the presence of 200 µMdUTP. For example, 1 µl of appropriately diluted bacterial extract (i.e., heat-treated and clarified extract of bacterial cells expressing a cloned polymerase or mutated cloned polymerase) is added to 10 µl of each nucleotide cocktail (200µM dATP, 200µM dGTP, 200µM dCTP and 5 µCi/ml α-^{33}P dCTP, ^{3}H-dCTP and 200µM dUTP or 200µM dTTP, activated calf thymus DNA, 1X appropriate buffer (see above)), followed by incubation at the optimal temperature for 30 minutes (e.g., 73°C for Pfu DNA polymerase), for example, as described in Hogrefe et al., 2001, Methods in Enzymology, 343:91-116. Extension reactions are then quenched on ice, and 5µl aliquots are spotted immediately onto DE81 ion-exchange filters (2.3cm; Whatman #3658323). Unincorporated label is removed by 6 washes with 2 x SCC (0.3M NaCl, 30mM sodium

citrate, pH 7.0), followed by a brief wash with 100% ethanol. Incorporated radioactivity is then measured by scintillation counting. Reactions that lack enzyme are also set up along with sample incubations to determine "total cpms" (omit filter wash steps) and "minimum cpms"(wash filters as above). Cpms bound is proportional to the amount of polymerase activity present per volume of bacterial extract. Mutants that can incorporate significant radioactivity in the presence of dUTP are selected for further analysis.

Mutant DNA polymerases with reduced uracil recognition can also be identified as those that can synthesize PCR products in the presence of 100% dUTP(See Example 3).

The "uracil detection" activity can also be determined using the long range primer extension assay on single uracil templates as described by Greagg et al., (1999) Proc. Natl. Acad. Sci. 96, 9045-9050. Briefly, the assay requires a 119- mer template that is generated by PCR amplification of a segment of pUC19 spanning the polylinker cloning site. PCR primer sequences are:

A, GACGTTGTAAAACGACGGCCAGU (SEQ ID NO: 3);

B, ACGTTGTAAAACGACGGCCAGT (SEQ ID NO: 4); and

C, CAATTTCACACAGGAAACAGCTATGACCATG (SEQ ID NO: 5).

The 119- mer oligonucleotide incorporating either a U or T nucleotide 23 bases from the terminus of one strand, was synthesized by using Taq polymerase under standard PCR conditions, using primer C and either primer A or primer B. PCR products are then purified on agarose gels and extracted by using Qiagen columns.

For long range primer extension, primer C is annealed to one strand of the 119- bp PCR product by heating to 65 ^{0}C in reaction buffer and cooling to room temperature. The dNTPs, [α-[^{32}P] dATP, and 5 units of DNA polymerase (Pfu, Taq and mutant Pfu DNA polymerase to be tested) are added in polymerase reaction buffer (as specified by the suppliers of each polymerase) to a final volume of 20 µl, and the reaction is allowed to proceed for 60 min at 55 ^{0}C. Reaction products are subjected to electrophoresis in a denaturing acrylamide gel and scanned and recorded on a Fuji FLA- 2000 phosphorimager.

The ability of the DNA polymerases from the thermophilic archaea Pyrococcus furiosus (Pfu) and the test mutant Pfu DNA polymerase to extend a primer across a template containing a single deoxyuridine can then be determined and directly compared.

The 3' to 5' exonuclease activity of purified Archaeal DNA polymerase (e.g., Pfu, KOD, or JDF-3 DNA polymerase) may be assayed according to methods known in the art, e.g., as described herein above, and in U.S. Patent No. 5,489,523, incorporated herein by reference.

For example, a sample containing 0.01 to 0.1 unit of DNA polymerase activity is admixed in a 25 µl exonuclease reaction admixture containing 40 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 2.5 µg of Taq I restriction endonuclease-digested Lambda DNA fragments filled in with ^{3}H-dGTP and ^{3}H-dCTP. The labelled DNA substrate was prepared by digesting 1 mg lambda gt10 with 1000 units Taq I at 68°C for 3 hrs in 1X Universal Buffer (Stratagene), followed by filling in the 3' recessed ends with 25 µCi each of ^{3}H -dGTP and ^{3}H -dCTP using 50 units of Sequenase (USB; United States Biochemicals, Inc.); the labelled fragments were separated from unincorporated nucleotides by passage through a NucTrap column (Stratagene) following the manufacturer's instructions. After a 30 min incubation of the endonuclease reaction admixture at 72°C, the reaction was terminated by addition of 5 µl of 15 mg/ml BSA and 13 µl of 50% trichloroacetic acid, and incubated on ice for 30 min to precipitate the nucleic acids. The precipitated nucleic acids were then centrifuged at 9000xg for 5 min, and 25 µl of the resulting supernatant was removed for scintillation counting. All reactions were performed in triplicate. One unit of exonuclease activity catalyzes the acid solubilization of 10 nmole of total nucleotides in 30 min at 72°C.

The polymerization activity of any of the above enzymes can be defined by means well known in the art. One unit of DNA polymerization activity of conventional DNA polymerase, according to the subject invention, is defined as the amount of enzyme which catalyzes the incorporation of 10 nmoles of total deoxynucleotides (dNTPs) into polymeric form in 30 minutes at optimal temperature (e.g., 72°C for Pfu DNA polymerase).

IV. EXPRESSION OF WILD-TYPE OR MUTANT ENZYMES ACCORDING TO THE INVENTION

Methods known in the art may be applied to express and isolate the mutated forms of DNA polymerase (i.e., the second enzyme) according to the invention. The methods described here can be also applied for the expression of wild-type enzymes useful (e.g., the first enzyme) in the invention. Many bacterial expression vectors contain sequence elements or combinations of sequence elements allowing high level inducible expression of the protein encoded by a foreign sequence. For example, as mentioned above, bacteria expressing an integrated inducible form of the T7 RNA polymerase gene may be transformed with an expression vector bearing a mutated DNA polymerase gene linked to the T7 promoter. Induction of the T7 RNA polymerase by addition of an appropriate inducer, for example, isopropyl-β-D-thiogalactopyranoside (IPTG) for a lac-inducible promoter, induces the high level expression of the mutated gene from the T7 promoter.

Appropriate host strains of bacteria may be selected from those available in the art by one of skill in the art. As a non-limiting example, E. coli strain BL-21 is commonly used for expression of exogenous proteins since it is protease deficient relative to other strains of E. coli. BL-21 strains bearing an inducible T7 RNA polymerase gene include WJ56 and ER2566 (Gardner & Jack, 1999, supra). For situations in which codon usage for the particular polymerase gene differs from that normally seen in E. coli genes, there are strains of BL-21 that are modified to carry tRNA genes encoding tRNAs with rarer anticodons (for example, argU, ileY, leuW, and proL tRNA genes), allowing high efficiency expression of cloned protein genes, for example, cloned Archaeal enzyme genes (several BL21-CODON PLUSTM cell strains carrying rare-codon tRNAs are available from Stratagene, for example).

There are many methods known to those of skill in the art that are suitable for the purification of a modified DNA polymerase of the invention. For example, the method of Lawyer et al. (1993, PCR Meth. & App. 2: 275) is well suited for the isolation of DNA polymerases expressed in E. coli, as it was designed originally for the isolation of Taq polymerase. Alternatively, the method of Kong et al. (1993, J. Biol. Chem. 268: 1965, incorporated herein by reference) may be used, which employs a heat denaturation step to

destroy host proteins, and two column purification steps (over DEAE-Sepharose and heparin-Sepharose columns) to isolate highly active and approximately 80% pure DNA polymerase. Further, DNA polymerase mutants may be isolated by an ammonium sulfate fractionation, followed by Q Sepharose and DNA cellulose columns, or by adsorption of contaminants on a HiTrap Q column, followed by gradient elution from a HiTrap heparin column.

The invention further provides for mutant V93R, V93E, V93D, V93K or V93N Pfu DNA polymerases that contain one or more additional mutations with improved reverse transcriptase activity, as described in U.S. Application with Serial No. 10/435,766, hereby incorporated by reference.

V. DNA polymerase blend and PCR additives

The invention further provides for compositions in which any of the Archaeal mutant DNA polymerases are mixed with either a second DNA polymerase (either wild type or another mutant DNA polymerase). For example, a mutant DNA polymerase with deficient 3'-5' exonuclease activity and reduced uracil detection activity (or additionally with increased reverse transcriptase activity) may be mixed with:

a.) an Archaeal DNA polymerase with reduced polymerization activity

b) a wild type DNA polymerase with no 3'-5' exonuclease activity, e.g., Taq polymerase

c) a polymerase chimera (e.g., Pfu chimera as as described in WO 01/92501 A1 or Pavlov et al. supra)

d) a reverse transcriptase, such as HIV, HTLV-I, HTLV-II, FeLV, FIV, SIV, AMV, MMTV, and MoMuLV reverse transcriptases.

The present invention also provides a composition containing one mutant archaeal DNA polymerase with no 3'-5' exonuclease activity and another mutant archaeal DNA polymerase with 3'-5' exonuclease activity.

Preferably, both the mutant archaeal DNA polymerase with no 3'-5' exonuclease activity and the other mutant archaeal DNA polymerase with 3'-5' exonuclease activity contain a mutation at V93.

The present invention also provides compositions which contain the mutant DNA polymerase and an PCR additive, such as one or more selected from the group consisting of: Pfu dUTPase (PEF), PCNA, RPA, ssb, antibodies, DMSO, betaine, 3'-5' exonuclease (e.g., Pfu G387P), Ncp7, recA, and T4gp32, e.g., as described in U.S. Patent Application with Serial No. 20020119467, hereby incorporated by reference in its entirety.

The addition of NCp7 to a reverse transcription reaction, significantly increases the processivity of the reverse transcriptase enzyme. Hence, it is expected that a number of other general RNA binding proteins will have the same effect. Non-limiting examples of such RNA binding proteins, include nucleocapsid proteins from other retroviruses (Ncp7 is derived from HIV-1), p50 (a protein which possesses strong, but non-specific, RNA-binding activity and is associated with cytoplasmic mRNA), the FRGY 2 protein from Xenopus oocytes, La antigen, and polypyrimidine tract binding protein (hnRNP I/PTB) (Ghetti et al., 1992 Nucl. Acid. Res. 20: 3671-3678; Dreyfuss et al., 1993, Annu. Rev. Biochem. 62: 289-321; Chang et al., 1994, J. Virol. 68:7008-7020; and Spirin, 1998, In Hershey et al., (Eds), Translational Control, Cold Spring Harbor Laboratory press, Cold Spring Harbor, N.Y. pp. 319-334).

Similarly, although the improvement in the processivity of a RNA-dependent polymerase has been demonstrated with reverse transcriptase, the present invention should not be so limited. A recent report has demonstrated that a single missense mutation with the catalytic fragment of Moloney murine leukemia virus (MMLV) RT (the parental RT from which superscript is derived) is sufficient to convert this enzyme from a RNA-dependent DNA polymerase to a RNA-dependent RNA polymerase (Giao et al., 1997, Proc. Nati. Acad. Sci. USA 94: 407-411). It is thus expected that general RNA binding proteins will also stimulate the processivity of RNA-dependent RNA polymerases given that the inhibitory features of "difficult" RNA template will be present. Other examples of RNA-dependent RNA polymerases include the polymerases of all members of the picomavirus family which copy their mRNAs directly into ds RNA genome from a single stranded mRNA template.

In addition, it is expected that general DNA binding proteins will stimulate the processivity of DNA-dependent DNA polymerases and DNA-dependent RNA polymerase. While the methods of the instant invention have been demonstrated with rec A protein and single-strand DNA binding protein (SSB), other general DNA binding proteins could also be used as stimulators. A non-limiting example of a general DNA binding protein is the gene 32 product of T4 bacteriophage (T4gp32). Hence, it is expected that a number of other general DNA binding proteins will be able to stimulate, for example, T7DNA polymerase processivity during second strand synthesis when generating a cDNA library. Non-limiting examples of other general DNA binding proteins, include: ssCRE-BP/Pur.varies. (a protein isolated from rat lung); Hbsu (an essential nucleoid-associated protein from Bacillus subtilis); uvs.sup.y (a gene product of bacteriophage T4); replication protein A (a heterotrimeric ss DNA binding protein in eukaryotes); the BALF2 gene product of Epstein-Barr virus; the yeast RAD51 gene product; the SSB of Bacillus subtilis phage phi 29; and the SSB of adenovirus (Wei et al., 1998, Ipn. J. Pharmacol. 78: 418-42; Kohler et al., 1998, Mol. Gen. Genet .260: 487-491; Sweezy et al., 1999, Biochemistry 38: 936-944; Brill et al., 1998, Mol. Cel. Biol. 18 :7225-7234; Tsurumi et al., 1998, J. Gen. Virol, 79 :1257-1264; Namsaraev et al., 1997, Mol. Cell. Biol. 17: 5359-5368; Soengas et al., 1997, J. Biol. Chem. 272: 303-310; and Kanellopoulos et al., 1995, J. Struct. Biol. 115: 113-116).

In addition non-limiting examples of DNA-dependent DNA polymerases which could benefit from the processivity enhancing methods and compositions of the present invention include E. coli DNA polymerase, the klenow fragment of E. coli DNA polymerase, Vent polymerase, Pfu polymerase, Bst DNA polymerase, and any other thermophilic DNA polymerase. Also, as pertaining to CDNA systhesis, E. coli DNA polymerase (see FIG. 1), T4 DNA polymerase, and thermophilic DNA polymerases have all been used to generate second strand product depending on the strategy being undertaken (In cDNA Library Protocols, 1997, Cowell et al., (eds). Humana Press, Totowa, N.J.).

In addition, a composition containing the mutant DNA polymerase of the present invention may also contain additives like antibodies for increased specificity (for hot start PCR, described in Borns et al. (2001) Strategies 14, pages 5-8 and also in manual accompanying commercially available kit, Stratagene Catalogue # 600320), DMSO for GC-

rich PCR or single stranded DNA binding protein for higher specificity (commercially available, Stratagene Catalog # 600201), dUTP and/or uracil N-glycosylase.

V. APPLICATIONS OF THE SUBJECT INVENTION

In one aspect, the invention provides a method for DNA synthesis using the compositions of the subject invention. Typically, synthesis of a polynucleotide requires a synthesis primer, a synthesis template, polynucleotide precursors for incorporation into the newly synthesized polynucleotide, (e.g. dATP, dCTP, dGTP, dTTP), and the like. Detailed methods for carrying out polynucleotide synthesis are well known to the person of ordinary skill in the art and can be found, for example, in Molecular Cloning second edition, Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

A. APPLICATION IN AMPLIFICATION REACTIONS

"Polymerase chain reaction" or "PCR" refers to an in vitro method for amplifying a specific polynucleotide template sequence. The technique of PCR is described in numerous publications, including, PCR: A Practical Approach, M. J. McPherson, et al., IRL Press (1991), PCR Protocols: A Guide to Methods and Applications, by Innis, et al., Academic Press (1990), and PCR Technology: Principals and Applications for DNA Amplification, H. A. Erlich, Stockton Press (1989). PCR is also described in many U.S. Patents, including U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; 4,965,188; 4,889,818; 5,075,216; 5,079,352; 5,104,792; 5,023,171; 5,091,310; and 5,066,584, each of which is herein incorporated by reference.

For ease of understanding the advantages provided by the present invention, a summary of PCR is provided. The PCR reaction involves a repetitive series of temperature cycles and is typically performed in a volume of 50-100 µl. The reaction mix comprises dNTPs (each of the four deoxynucleotides dATP, dCTP, dGTP, and dTTP), primers, buffers, DNA polymerase, and polynucleotide template. PCR requires two primers that hybridize with the double-stranded target polynucleotide sequence to be amplified. In PCR, this double-stranded target sequence is denatured and one primer is annealed to each strand of the denatured target. The primers anneal to the target polynucleotide at sites removed from one

another and in orientations such that the extension product of one primer, when separated from its complement, can hybridize to the other primer. Once a given primer hybridizes to the target sequence, the primer is extended by the action of a DNA polymerase. The extension product is then denatured from the target sequence, and the process is repeated.

In successive cycles of this process, the extension products produced in earlier cycles serve as templates for DNA synthesis. Beginning in the second cycle, the product of amplification begins to accumulate at a logarithmic rate. The amplification product is a discrete double-stranded DNA molecule comprising: a first strand which contains the sequence of the first primer, eventually followed by the sequence complementary to the second primer, and a second strand which is complementary to the first strand.

Due to the enormous amplification possible with the PCR process, small levels of DNA carryover from samples with high DNA levels, positive control templates or from previous amplifications can result in PCR product, even in the absence of purposefully added template DNA. If possible, all reaction mixes are set up in an area separate from PCR product analysis and sample preparation. The use of dedicated or disposable vessels, solutions, and pipettes (preferably positive displacement pipettes) for RNA/DNA preparation, reaction mixing, and sample analysis will minimize cross contamination. See also Higuchi and Kwok, 1989, Nature, 339:237-238 and Kwok, and Orrego, in: Innis et al. eds., 1990, PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., San Diego, Calif., which are incorporated herein by reference.

The enzymes provided herein are also useful for dUTP/UNG cleanup methods that require PCR enzymes that incorporate dUTP (Longo et al., Supra).

In addition, Mutations that reduce uracil sensitivity are expected to improve the success rate of long-range amplification (higher yield, longer targets amplified). It is expected that mutations eliminating uracil detection will also increase the error rate of Archaeal DNA polymerases. If uracil stalling contributes to fidelity by preventing synthesis opposite promutagenic uracil (arising from cytosine deamination), then uracil insensitive mutants are likely to exhibit a higher GC→TA transition mutation rate. It is therefore envisioned that optimal PCR performance and fidelity may be achieved by adding to uracil-

insensitive Archaeal DNA polymerase mutants either thermostable exonucleases (e.g., polymerase reduced proofreading DNA polymerases, exonuclease III) or additional mutations that increase fidelity.

1. THERMOSTABLE ENZYMES

For PCR amplifications, the enzymes used in the invention are preferably thermostable. As used herein, "thermostable" refers to an enzyme which is stable to heat, is heat resistant, and functions at high temperatures, e.g., 50 to 90°C. The thermostable enzyme according to the present invention must satisfy a single criterion to be effective for the amplification reaction, i.e., the enzyme must not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded polynucleotides. By "irreversible denaturation" as used in this connection, is meant a process bringing a permanent and complete loss of enzymatic activity. The heating conditions necessary for denaturation will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the polynucleotides being denatured, but typically range from 85°C, for shorter polynucleotides, to 105°C for a time depending mainly on the temperature and the polynucleotide length, typically from 0.25 minutes for shorter polynucleotides, to 4.0 minutes for longer pieces of DNA. Higher temperatures may be tolerated as the buffer salt concentration and/or GC composition of the polynucleotide is increased. Preferably, the enzyme will not become irreversibly denatured at 90 to 100°C. An enzyme that does not become irreversibly denatured, according to the invention, retains at least 10%, or at least 25%, or at least 50% or more function or activity during the amplification reaction.

2. PCR REACTION MIXTURE

In addition to the subject enzyme mixture, one of average skill in the art may also employ other PCR parameters to increase the fidelity of synthesis/amplification reaction. It has been reported PCR fidelity may be affected by factors such as changes in dNTP concentration, units of enzyme used per reaction, pH, and the ratio of Mg^{2+} to dNTPs present in the reaction (Mattila et al., 1991, supra).

Mg^{2+} concentration affects the annealing of the oligonucleotide primers to the template DNA by stabilizing the primer-template interaction, it also stabilizes the replication complex of polymerase with template-primer. It can therefore also increases non-specific annealing and produced undesirable PCR products (gives multiple bands in gel). When non-specific amplification occurs, Mg^{2+} may need to be lowered or EDTA can be added to chelate Mg^{2+} to increase the accuracy and specificity of the amplification.

Other divalent cations such as Mn^{2+}, or Co^{2+} can also affect DNA polymerization. Suitable cations for each DNA polymerase are known in the art (e.g., in DNA Replication 2nd edition, supra). Divalent cation is supplied in the form of a salt such $MgCl_2$, $Mg(OAc)_2$, $MgSO_4$, $MnCl_2$, $Mn(OAc)_2$, or $MnSO_4$. Usable cation concentrations in a Tris-HCl buffer are for $MnCl_2$ from 0.5 to 7 mM, preferably, between 0.5 and 2 mM, and for $MgCl_2$ from 0.5 to 10 mM. Usable cation concentrations in a Bicine/KOAc buffer are from 1 to 20 mM for $Mn(OAc)_2$, preferably between 2 and 5 mM.

Monovalent cation required by DNA polymerase may be supplied by the potassium, sodium, ammonium, or lithium salts of either chloride or acetate. For KCl, the concentration is between 1 and 200 mM, preferably the concentration is between 40 and 100 mM, although the optimum concentration may vary depending on the polymerase used in the reaction.

Deoxyribonucleotide triphosphates (dNTPs) are added as solutions of the salts of dATP, dCTP, dGTP, dUTP, and dTTP, such as disodium or lithium salts. In the present methods, a final concentration in the range of 1 μM to 2 mM each is suitable, and 100-600 μM is preferable, although the optimal concentration of the nucleotides may vary in the PCR reaction depending on the total dNTP and divalent metal ion concentration, and on the buffer, salts, particular primers, and template. For longer products, i.e., greater than 1500 bp, 500 μM each dNTP may be preferred when using a Tris-HCl buffer.

dNTPs chelate divalent cations, therefore amount of divalent cations used may need to be changed according to the dNTP concentration in the reaction. Excessive amount of dNTPs (e.g., larger than 1.5 mM) can increase the error rate and possibly inhibit DNA polymerases. Lowering the dNTP (e.g., to 10-50 μM) may therefore reduce error rate. PCR reaction for amplifying larger size template may need more dNTPs.

One suitable buffering agent is Tris-HCl, preferably pH 8.3, although the pH may be in the range 8.0-8.8. The Tris-HCl concentration is from 5-250 mM, although 10-100 mM is most preferred. A preferred buffering agent is Bicine-KOH, preferably pH 8.3, although pH may be in the range 7.8-8.7. Bicine acts both as a pH buffer and as a metal buffer. Tricine may also be used.

PCR is a very powerful tool for DNA amplification and therefore very little template DNA is needed. However, in some embodiments, to reduce the likelihood of error, a higher DNA concentration may be used, though too many templates may increase the amount of contaminants and reduce efficiency.

Usually, up to 3 μM of primers may be used, but high primer to template ratio can results in non-specific amplification and primer-dimer formation. Therefore it is usually necessary to check primer sequences to avoid primer-dimer formation.

The invention provides for Pfu V93R, V93E, V93K , V93D , or V93N DNA polymerases with reduced uracil detection activity that enhance PCR of GC rich DNA templates by minimizing the effect of cytosine deamination in the template and by allowing the use of higher denaturation times and denaturation temperatures.

3. CYCLING PARAMETERS

Denaturation time may be increased if template GC content is high. Higher annealing temperature may be needed for primers with high GC content or longer primers. Gradient PCR is a useful way of determining the annealing temperature. Extension time should be extended for larger PCR product amplifications. However, extension time may need to be reduced whenever possible to limit damage to enzyme.

The number of cycle can be increased if the number of template DNA is very low, and decreased if high amount of template DNA is used.

4. PCR ENHANCING FACTORS AND ADDITIVES

PCR enhancing factors may also be used to improve efficiency of the amplification. As used herein, a "PCR enhancing factor" or a "Polymerase Enhancing Factor" (PEF) refers to a complex or protein possessing polynucleotide polymerase enhancing activity (Hogrefe et al., 1997, Strategies 10::93-96; and U.S. Patent No. 6,183,997, both of which are hereby incorporated by references). For Pfu DNA polymerase, PEF comprises either P45 in native form (as a complex of P50 and P45) or as a recombinant protein. In the native complex of Pfu P50 and P45, only P45 exhibits PCR enhancing activity. The P50 protein is similar in structure to a bacterial flavoprotein. The P45 protein is similar in structure to dCTP deaminase and dUTPase, but it functions only as a dUTPase converting dUTP to dUMP and pyrophosphate. PEF, according to the present invention, can also be selected from the group consisting of: an isolated or purified naturally occurring polymerase enhancing protein obtained from an archeabacteria source (e.g., *Pyrococcus furiosus*); a wholly or partially synthetic protein having the same amino acid sequence as Pfu P45, or analogs thereof possessing polymerase enhancing activity; polymerase-enhancing mixtures of one or more of said naturally occurring or wholly or partially synthetic proteins; polymerase-enhancing protein complexes of one or more of said naturally occurring or wholly or partially synthetic proteins; or polymerase-enhancing partially purified cell extracts containing one or more of said naturally occurring proteins (U.S. Patent No. 6,183,997, supra). The PCR enhancing activity of PEF is defined by means well known in the art. The unit definition for PEF is based on the dUTPase activity of PEF (P45), which is determined by monitoring the production of pyrophosphate (PPi) from dUTP. For example, PEF is incubated with dUTP (10mM dUTP in 1x cloned Pfu PCR buffer) during which time PEF hydrolyzes dUTP to dUMP and PPi. The amount of PPi formed is quantitated using a coupled enzymatic assay system that is commercially available from Sigma (#P7275). One unit of activity is functionally defined as 4.0 nmole of PPi formed per hour (at 85°C).

Other PCR additives may also affect the accuracy and specificity of PCR reaction. EDTA less than 0.5 mM may be present in the amplification reaction mix. Detergents such as Tween-20™ and Nonidet™ P-40 are present in the enzyme dilution buffers. A final concentration of non-ionic detergent approximately 0.1% or less is appropriate, however, 0.01-0.05% is preferred and will not interfere with polymerase activity. Similarly, glycerol is often present in enzyme preparations and is generally diluted to a concentration of 1-20% in

the reaction mix. Glycerol (5-10%), formamide (1-5%) or DMSO (2-10%) can be added in PCR for template DNA with high GC content or long length (e.g., > 1kb). These additives change the Tm (melting temperature) of primer-template hybridization reaction and the thermostability of polymerase enzyme. BSA (up to 0.8 μg/μl) can improve efficiency of PCR reaction. Betaine (0.5-2M) is also useful for PCR over high GC content and long fragments of DNA. Tetramethylammonium chloride (TMAC, >50mM), Tetraethylammonium chloride (TEAC), and Trimethlamine N-oxide (TMANO) may also be used. Test PCR reactions may be performed to determine optimum concentration of each additive mentioned above.

The invention provides for additive including, but not limited to antibodies (for hot start PCR) and ssb (higher specificity). The invention also contemplates mutant ARCHAEAL DNA polymerases in combination with accessory factors, for example as described in U.S. 6,333,158, and WO 01/09347 A2, hereby incorporated by reference in its entirety.

Various specific PCR amplification applications are available in the art (for reviews, see for example, Erlich, 1999, Rev Immunogenet., 1:127-34; Prediger 2001, Methods Mol. Biol. 160:49-63; Jurecic et al., 2000, Curr. Opin. Microbiol. 3:316-21; Triglia, 2000, Methods Mol. Biol. 130:79-83; MaClelland et al., 1994, PCR Methods Appl. 4:S66-81; Abramson and Myers, 1993, Current Opinion in Biotechnology 4:41-47; each of which is incorporated herein by references).

The subject invention can be used in PCR applications including, but are not limited to, i) hot-start PCR which reduces non-specific amplification; ii) touch-down PCR which starts at high annealing temperature, then decreases annealing temperature in steps to reduce non-specific PCR product; iii) nested PCR which synthesizes more reliable product using an outer set of primers and an inner set of primers; iv) inverse PCR for amplification of regions flanking a known sequence. In this method, DNA is digested, the desired fragment is circularized by ligation, then PCR using primer complementary to the known sequence extending outwards; v) AP-PCR (arbitrary primed)/RAPD (random amplified polymorphic DNA). These methods create genomic fingerprints from species with little-known target sequences by amplifying using arbitrary oligonucleotides; vi) RT-PCR which uses RNA-directed DNA polymerase (e.g., reverse transcriptase) to synthesize cDNAs which is then

used for PCR. This method is extremely sensitive for detecting the expression of a specific sequence in a tissue or cells. It may also be use to quantify mRNA transcripts; vii) RACE (rapid amplification of cDNA ends). This is used where information about DNA/protein sequence is limited. The method amplifies 3' or 5' ends of cDNAs generating fragments of cDNA with only one specific primer each (plus one adaptor primer). Overlapping RACE products can then be combined to produce full length cDNA; viii) DD-PCR (differential display PCR) which is used to identify differentially expressed genes in different tissues. First step in DD-PCR involves RT-PCR, then amplification is performed using short, intentionally nonspecific primers; ix) Multiplex-PCR in which two or more unique targets of DNA sequences in the same specimen are amplified simultaneously. One DNA sequence can be use as control to verify the quality of PCR; x) Q/C-PCR (Quantitative comparative) which uses an internal control DNA sequence (but of different size) which compete with the target DNA (competitive PCR) for the same set of primers; xi) Recusive PCR which is used to synthesize genes. Oligonucleotides used in this method are complementary to stretches of a gene (>80 bases), alternately to the sense and to the antisense strands with ends overlapping (~20 bases); xii) Asymmetric PCR; xiii) In Situ PCR; xiv) Site-directed PCR Mutagenesis.

It should be understood that this invention is not limited to any particular amplification system. As other systems are developed, those systems may benefit by practice of this invention.

B. APPLICATION IN QUANTITATIVE PCR AND QUANTITATIVE RT-PCR

A typical PCR reaction includes multiple amplification steps, or cycles that selectively amplify a target nucleic acid species. A full description of the PCR process, and common variations thereof, such as quantitative PCR (QPCR), real-time QPCR, reverse transcription PCR (RT-PCR) and quantitative reverse transcription PCR (QRT-PCR) is beyond the scope of this disclosure and these methods are well-described in the art and have been broadly commercialized.

The present invention may be used to perform any of the above PCR methods known in the art (e.g., as reviewed in Joyce et al. (2002, Methods Mol Biol. 193:83-92), Klein (2002,

Trends Mol Med. 8(6):257-60), Wittwer et al. (2001, Methods. 25(4):430-42), Freeman et al. (1999, Biotechniques. 26(1):112-22, 124-5), hereby incorporated by reference.

Reverse transcription of an RNA template into cDNA is an integral part of many techniques used in molecular biology. Accordingly, the reverse transcription procedures, compositions, and kits provided in the present invention find a wide variety of uses. For example, it is contemplated that the reverse transcription procedures and compositions of the present invention are utilized to produce cDNA inserts for cloning into cDNA library vectors (e.g., lambda gt10 [Huynh et al., In DNA Cloning Techniques: A Practical Approach, D. Glover, ed., IRL Press, Oxford, 49, 1985], lambda gt11 [Young and Davis, Proc. Nat'l. Acad. Sci., 80:1194, 1983], pBR322 [Watson, Gene 70:399-403, 1988], pUC19 [Yarnisch-Perron et al., Gene 33:103-119, 1985], and M13 [Messing et al., Nucl. Acids. Res. 9:309-321, 1981]). The present invention also finds use for identification of target RNAs in a sample via RT-PCR (e.g., U.S. Pat. No. 5,322,770, incorporated herein by reference). Additionally, the present invention finds use in providing cDNA templates for techniques such as differential display PCR (e.g., Liang and Pardee, Science 257(5072):967-71 (1992). The DNA polymerase with increased RT activity, compositions or kits comprising such polymerase can be applied in any suitable applications, including, but not limited to the following examples.

1. *Reverse Transcription*

The present invention contemplates the use of thermostable DNA polymerase for reverse transcription reactions. Accordingly, in some embodiments of the present invention, thermostable DNA polymerases having increased RT activity are provided. In some embodiments, the thermostable DNA polymerase is selected from the DNA polymerases listed in Tables II-IV, for example, a Pfu or a JDF-3 DNA polymerase.

In some embodiments of the present invention, where a DNA polymerase with increased RT activity is utilized to reverse transcribe RNA, the reverse transcription reaction is conducted at about 50°C to 80°C, preferably about 60°C to 75°C. Optimal reaction temperature for each DNA polymerase is know in the art and may be relied upon as the optimal temperature for the mutant DNA polymerases of the present invention. Preferred

conditions for reverse transcription are 1X MMLV RT buffer (50 mM Tris pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$), containing 20% DMSO.

In still further embodiments, reverse transcription of an RNA molecule by a DNA polymerase with increased RT activity results in the production of a cDNA molecule that is substantially complementary to the RNA molecule. In other embodiments, the DNA polymerase with increased RT activity then catalyzes the synthesis of a second strand DNA complementary to the cDNA molecule to form a double stranded DNA molecule. In still further embodiments of the present invention, the DNA polymerase with increased RT activity catalyzes the amplification of the double stranded DNA molecule in a PCR as described below. In some embodiments, PCR is conducted in the same reaction mix as the reverse transcriptase reaction (i.e., a single tube reaction is performed). In other embodiments, PCR is performed in a separate reaction mix on an aliquot removed from the reverse transcription reaction (i.e., a two tube reaction is performed).

In another embodiment, the DNA polymerase mutants of the invention can be used for labeling cDNA for microarray analysis, e.g., with fluorescent labels such as Cy3, Cy5 or other labels. It is contemplated that DNA polymerase mutants as described herein would have the advantage of more efficient labeling or more uniform incorporation of labeled nucleotides relative to wild-type enzymes.

2. *QPCR and RT_QPCR*

The mutant DNA polymerase of the present invention is generally applicable to QPCR or RT-QPCR.

A quantitative reverse transcriptase polymerase chain reaction (RT-QPCR) method is provided for rapidly and accurately detecting low abundance RNA species in a population of RNA molecules (for example, and without limitation, total RNA or mRNA), including the steps of: a) incubating an RNA sample with a reverse transcriptase and a high concentration of a target sequence-specific reverse transcriptase primer under conditions suitable to generate cDNA; b) subsequently adding suitable polymerase chain reaction (PCR) reagents to the reverse transcriptase reaction, including a high concentration of a PCR primer set specific to

the cDNA and a thermostable DNA polymerase to the reverse transcriptase reaction, and c) cycling the PCR reaction for a desired number of cycles and under suitable conditions to generate PCR product ("amplicons") specific to the cDNA. By temporally separating the reverse transcriptase and the PCR reactions, and by using reverse transcriptase-optimized and PCR-optimized primers, excellent specificity is obtained. The reaction is conducted in a single tube (all tubes, containers, vials, cells and the like in which a reaction is performed may be referred to herein, from time to time, generically, as a "reaction vessel"), removing a source of contamination typically found in two-tube reactions. The high concentration primers permit very rapid QRT-PCR reactions, typically on the order of 20 minutes from the beginning of the reverse transcriptase reaction to the end of a 40 cycle PCR reaction. The realization of such a rapid QRT-PCR experiment is assisted by the availability of thermal cycling devices capable of generating a thermal ramp rate (delta T) of at least about 5 °C per second.

The reaction c) may be performed in the same tube as the reverse transcriptase reaction by adding sufficient reagents to the reverse transcriptase (RT) reaction to create good, or even optimal conditions for the PCR reaction to proceed. A single tube may be loaded, prior to the running of the reverse transcriptase reaction, with: 1) the reverse transcriptase reaction mixture, and 2) the PCR reaction mixture to be mixed with the cDNA mixture after the reverse transcriptase reaction is completed. The reverse transcriptase reaction mixture and the PCR reaction mixture may be physically separated by a solid, or semi-solid (including amorphous, glassy substances and waxy) barrier of a composition that melts at a temperature greater than the incubation temperature of the reverse transcriptase reaction, but below the denaturing temperature of the PCR reaction. The barrier composition may be hydrophobic in nature and forms a second phase with the RT and PCR reaction mixtures when in liquid form. One example of such a barrier composition is wax beads, commonly used in PCR reactions, such as the AMPLIWAX PCR GEM products commercially available from Applied Biosystems of Foster City, Calif. and the STRATASPHERE Magnesium Wax Beads, commercially available from Stratagene of La Jolla, Calif.

In one type of two-step process, the first step involves synthesis of first strand cDNA with a reverse transcriptase, following by a second PCR step. In certain protocols, these steps are carried out in separate reaction tubes. In these two tube protocols, following reverse transcription of the initial RNA template in the first tube, an aliquot of the resultant product is then placed into the second PCR tube and subjected to PCR amplification.

In a second type of two-step process, both RT and PCR are carried out in the same tube using a compatible RT and PCR buffer. Typically, reverse transcription is carried out first, followed by addition of PCR reagents to the reaction tube and subsequent PCR.

Reverse transcription is commonly performed with viral reverse transcriptases isolated from Avian myeloblastosis virus (AMV-RT) or Moloney murine leukemia virus (MMLV-RT), which are active in the presence of magnesium ions.

The mutant DNA polymerase may be used in performing two-step RT-QPCR, in which RT is performed by a conventional reverse transcriptase and the quantitative PCR is performed by a mutant DNA polymerase of the present invention.

A variety of one-step RT-PCR protocols have been developed, see Blain & Goff, J. Biol. Chem. (1993) 5: 23585-23592; Blain & Goff, J. Virol. (1995) 69:4440-4452; Sellner et al., J. Virol. Method. (1994) 49:47-58; PCR, Essential Techniques (ed. J. F. Burke, J. Wiley & Sons, New York)(1996) pp61-63; 80-81.

Some one-step systems are commercially available, for example, SuperScript One-Step RT-PCR System description on the world-wide web at lifetech.com/world_whatsn-ew/archive/nz_{1-3}.html; Access RT-PCR System and Access RT-PCR Introductory System described on the world wide web at promega.com/tbs/tb220/tb220.html; AdvanTaq & AdvanTaq Plus PCR kits and User Manual available at www.clontech.com, and ProSTAR™ HF single-tube RT-PCR kit (Stratagene, Catalog No. 600164, information available on the world wide web at stratagene.com).

Certain RT-PCR methods use an enzyme blend or enzymes with both reverse transcriptase and DNA polymerase or exonuclease activities, e.g., as described in U.S. Patent Nos. 6,468,775; 6,399,320; 5,310,652; 6,300,073; Patent Application No. U.S.

2002/0119465A1; EP 1,132,470A1 and WO 00/71739A1, all of which are incorporated herein by reference.

The reverse transcription and PCR may also be performed in a single step reaction using a mutant DNA polymerase of the present invention which also contains an increased reverse transcriptase activity.

As used herein, "quantitative PCR (QPCR)" refers to a PCR amplification which is used to determine the abundance of polynucleotide as described herein above. To determine the abundance of a specific polynucleotide present in a PCR reaction, this method usually utilizes a labeling dye which fluoresces in proportion to the amount of target DNA species that is produced by the PCR reaction.

According to one embodiment of the present invention, the quantitative PCR methods may amplify, in the presence of Mg ions, a target nucleic acid by using dATF, dGTP, dCTP, dTTP or dUTP, a target nucleic acid (DNA or RNA), a mutant DNA polymerase of the invention, a primer, and a nucleic acid labeled with a fluorescent dye or an intercalator while repeatedly changing the temperature between low and high levels, and monitor increases in fluorescence emission from the fluorescent dye in real time in the course of the amplification.

In the case of a fluorescent probe, the reaction fluoresces in relative proportion to the quantity of DNA product produced.

TaqMan is a homogenous assay for detecting polynucleotides (U.S. Patents 5,723,591). In this assay, two PCR primers flank a central probe oligonucleotide. The probe oligonucleotide contains two fluorescent moieties. During the polymerization step of the PCR process, the polymerase cleaves the probe oligonucleotide. The cleavage causes the two fluorescent moieties to become physically separated, which causes a change in the wavelength of the fluorescent emission. As more PCR product is created, the intensity of the novel wavelength increases. The TaqMan.TM. procedure (Applied Biosystems, CA) describes one such fluorescent methodology for performing Quantitative PCR. Briefly described, this system integrates the use of a detectable reporter construct, or probe, which comprises both a fluorescent label molecule and a quencher molecule. Ordinarily, the quencher nullifies the

majority of fluorescence which may be emitted by the probe. During the amplification process, however, the quencher molecule is released from the probe allowing the fluorescent label to be detected. The quantity or intensity of fluorescence may then be correlated with the amount of product formed in the reaction. Using this information, calculations can be made to determine the initial quantity of template present. Quantitation in this manner is useful in applications including: determination of levels/concentrations of specific DNA and RNA sequences in tissue samples, identification of viral loads, genotyping, and numerous other applications. For additional information regarding the fundamental concepts of quantitative PCR the reader is directed to Allelic Discrimination by Nick-Translation PCR with Fluorogenic Probes, L. G. Lee, C. R. Connell, and W. Bloch, Nucleic Acids Research 21:3761-3766, 1993 and PCR Technology: Principles and Applications for DNA Amplification. Karl Drlica, John Wiley and Sons, 1997.

Molecular beacons are an alternative to TaqMan (U.S. Patent Nos. 6,277,607; 6,150,097; 6,037,130) for the detection of polynucleotides. Molecular beacons are oligonucleotide hairpins which undergo a conformational change upon binding to a perfectly matched template. The conformational change of the oligonucleotide increases the physical distance between a fluorophore moiety and a quencher moiety present on the oligonucleotide. This increase in physical distance causes the effect of the quencher to be diminished, thus increasing the signal derived from the fluorophore.

U.S. Patent NO. 6,174,670B1 discloses methods of monitoring hybridization during a polymerase chain reaction which are achieved with rapid thermal cycling and use of double stranded DNA dyes or specific hybridization probes in the presence of a fluorescence resonance energy transfer pair — fluorescein and Cy5.3 or Cy5.5. The method amplifies the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of the probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor fluorophore interacts with the acceptor fluorophore to generate a detectable signal. The sample is then excited with light at a wavelength absorbed by the donor fluorophore and

the fluorescent emission from the fluorescence energy transfer pair is detected for the determination of that target amount.

There are also several other fluorescent and enzymatic PCR technologies, such as Scorpions™, Sunrise™ primers, and DNAzymes, for polynucleotide detection, where each polynucleotide to be detected requires a different oligonucleotide probe and two different fluorescent moieties.

In addition, QPCR may also be performed according to methods as described in U.S. Patent Application with Serial No. 60/435,484, hereby incorporated by reference in its entirety.

In one embodiment, the mutant DNA polymerase is used in a method for detecting the amount of a target polynucleotide in an amplification reaction mixture, comprising: (a) providing a forward and a reverse primer which amplify the target polynucleotide in the amplification reaction mixture; (b) providing to the reaction mixture a target-hybridizing probe 1 comprising a target binding sequence (P1-DNA) which hybridizes to one strand of the target polynucleotide and a probe binding sequence (P1-P) which does not hybridize to the target polynucleotide, and a target-hybridizing probe 2 comprising a target binding sequence (P2-DNA) which hybridizes, in close proximity, to the same strand of the target polynucleotide and a probe binding sequence (P2-P) which does not hybridize to the target polynucleotide; (c) providing to the reaction mixture a non-target-hybridizing universal probe 3 labeled with label A and a non-target-hybridizing universal probe 4 labeled with label B, where the universal probe 3 hybridize to the P1-P sequence and the universal probe 4 hybridizes to the P2-P sequence, and where the label A interact with the label B to generate a signal; and (d) detecting the generated signal which is indicative as to the amount of the polynucleotide in the sample.

C. APPLICATION IN DIRECT CLONING OF PCR AMPLIFIED PRODUCT

It is understood that the amplified product produced using the subject enzyme can be cloned by any method known in the art. In one embodiment, the invention provides a composition which allows direct cloning of PCR amplified product.

The most common method for cloning PCR products involves incorporation of flanking restriction sites onto the ends of primer molecules. The PCR cycling is carried out and the amplified DNA is then purified, restricted with an appropriate endonuclease(s) and ligated to a compatible vector preparation.

A method for directly cloning PCR products eliminates the need for preparing primers having restriction recognition sequences and it would eliminate the need for a restriction step to prepare the PCR product for cloning. Additionally, such method would preferably allow cloning PCR products directly without an intervening purification step.

U.S. Patent Nos. 5,827,657 and 5,487,993 (hereby incorporated by their entirety) disclose methods for direct cloning of PCR products using a DNA polymerase which takes advantage of the single 3'-deoxy-adenosine monophosphate (dAMP) residues attached to the 3' termini of PCR generated polynucleotides. Vectors are prepared with recognition sequences that afford single 3'-terminal deoxy-thymidine monophosphate (dTMP) residues upon reaction with a suitable restriction enzyme. Thus, PCR generated copies of genes can be directly cloned into the vectors without need for preparing primers having suitable restriction sites therein.

Taq DNA polymerase exhibits terminal transferase activity that adds a single dATP to the 3' ends of PCR products in the absence of template. This activity is the basis for the TA cloning method in which PCR products amplified with Taq are directly ligated into vectors containing single 3'dT overhangs. Archaeal DNA polymerase, on the other hand, lacks terminal transferase activity, and thus produces blunt-ended PCR products that are efficiently cloned into blunt-ended vectors.

In one embodiment, the invention provides for a PCR product, generated in the presence of a mutant DNA polymerase of the present invention, that is subsequently incubated with Taq DNA polymerase in the presence of dATP at 72°C for 15-30 minutes. Addition of 3'-dAMP to the ends of the amplified DNA product then permits cloning into TA cloning vectors according to methods that are well known to a person skilled in the art.

D. APPLICATION IN DNA SEQUENCING

The invention further provides for dideoxynucleotide DNA sequencing methods using thermostable DNA polymerases having a reduced base analog detection activity to catalyze the primer extension reactions. Methods for dideoxynucleotide DNA sequencing are well known in the art and are disclosed in U.S. Patent Nos. 5,075,216, 4,795,699 and 5,885,813, the contents of which are hereby incorporated in their entirety.

E. APPLICATION IN MUTAGENESIS

The mutant Archaeal DNA polymerases of the invention, preferably V93R Pfu DNA polymerase, also provide enhanced efficacy for PCR-based or linear amplification-based mutagenesis. The invention therefore provides for the use of the mutant Archaeal DNA polymerases with reduced base analog detection activity for site-directed mutagenesis and their incorporation into commercially available kits, for example, QuikChange Site-directed Mutagenesis, QuikChange Multi-Site-Directed Mutagenesis (Stratagene). Site-directed mutagenesis methods and reagents are disclosed in the pending U.S. Patent Application No. 10/198,449 (Hogrefe et al.; filed July 18, 2002), the contents of which are hereby incorporated in its entirety. The invention also encompasses Mutazyme (exo$^-$Pfu in combination with PEF, GeneMorph Kit). The GeneMorph kits are disclosed in the pending U.S. Patent Application No.: 10/154,206 (filed May 23, 2002), the contents of which are hereby incorporated in its entirety.

All of the mutant Archaeal DNA polymerases contemplated herein are useful for PCR and RT-PCR.

VI. KITS

The invention herein also contemplates a kit format which comprises a package unit having one or more containers of the subject composition and in some embodiments including containers of various reagents used for polynucleotide synthesis, including synthesis in PCR. The kit may also contain one or more of the following items: polynucleotide precursors, primers, buffers, instructions, and controls. Kits may include containers of reagents mixed together in suitable proportions for performing the methods in accordance with the invention.

Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

The invention contemplates a kit comprising a combination of a mutant ARCHAEAL DNA polymerase of the invention, and another mutant or wild type DNA polymerase.

The invention contemplates a kit comprising a combination of a mutant Archaeal DNA polymerase of the invention, and a PCR additive.

VII. EXAMPLES

Example 1. Construction of Tgo, Pfu, KOD or JDF-3 DNA Polymerase Mutants with deficient 3'-5' exonuclease activity and reduced Uracil Detection

In one embodiment of the invention, Tgo, Pfu, KOD or JDF-3 DNA polymerase mutants exhibiting substantially reduced 3'-5' exonuclease activity are prepared by introducing amino acid substitutions at the conserved 141D or 143E residues in the exo I domain. Using the CHAMELEON® Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene), the following mutants are constructed: D141A, D141N, D141S, D141T, D141E and E143A for Tgo, Pfu, KOD or JDF-3 DNA polymerases.

To analyze Tgo, Pfu, KOD, JDF-3 mutant proteins, the DNA sequence encoding each of Tgo, Pfu, KOD, and JDF-3 DNA polymerases is PCR amplified using primers GGG AAA **CAT ATG** ATC CTT GAC GTT GAT TAC (where NdeI site in bold and start codon underlined) and GGG AAA **GGA TCC** TCA CTT CTT CTT CCC CTT C (where BamHI site shown in bold type). The PCR products are digested, purified, and ligated into a high expression level vector using standard methods. Plasmid clones are transformed into BL21(DE3). Recombinant bacterial clones are grown using standard procedures and polymerase mutants are expressed in the absence of induction. The exonuclease and polymerase activities of recombinant clones are assayed using bacterial lysates. Typically, crude extracts are heated at 70°C for 15-30 minutes and then centrifuged to obtain a cleared lysate.

The combination exonuclease mutant D141A+E143A is also made as described above herein in the description.

The D141T, E143A, D141A or D141A+E143A double mutants which exhibits significantly reduced 3'-5' exo activity may be chosen for further mutagenesis. For experiment or applications requiring maximal elimination of 3' to 5' exonuclease activity, the double mutant D141A + E143A is preferred.

Additional mutations are introduced into Tgo, Pfu, KOD or JDF-3 DNA polymerase exo- mutants that are likely to reduce uracil detection, while having minimal effects on polymerase or proofreading activity. With the QuikChange Multi kit, specific point mutations (e.g., V93E, H, K, R, and N) are introduced by incorporating one phosphorylated mutagenic primer or by selecting random mutants from a library of Tgo, Pfu, KOD or JDF-3 DNA V93 variants, created by incorporating a degenerate codon (V93G and L). Clones are sequenced to identify the incorporated mutations.

For example, Valine 93 in Tgo, Pfu, KOD or JDF-3 DNA DNA polymerase may be substituted with Glycine (G), asparagine (N), arginine [R], glutamic acid (E), histidine (H), and leucine (L) using the QuikChange primer sequences listed in Figure 1.

Example 2. Preparation of Bacterial Extracts Containing Mutant Pfu, KOD or JDF-3 DNA Polymerases

Plasmid DNA is purified with the StrataPrep® Plasmid Miniprep Kit (Stratagene), and used to transform BL26-CodonPlus-RIL cells. Ampicillin resistant colonies are grown up in 1-5 liters of LB media containing Turbo Amp™ (100μg/μl) and chloramphenicol (30μg/μl) at 30°C with moderate aeration. The cells are collected by centrifugation and stored at -80°C until use.

Cell pellets (12-24 grams) are resuspended in 3 volumes of lysis buffer (buffer A: 50mM Tris HCl (pH 8.2), 1mM EDTA, and 10mM βME). Lysozyme (1 mg/g cells) and PMSF (1mM) were added and the cells were lysed for 1 hour at 4°C. The cell mixture is sonicated, and the debris removed by centrifugation at 15,000 rpm for 30 minutes (4°C).

Tween 20 and Igepal CA-630 are added to final concentrations of 0.1% and the supernatant is heated at 72°C for 10 minutes. Heat denatured *E. coli* proteins are then removed by centrifugation at 15,000 rpm for 30 minutes (4°C).

Example 3. Evaluate 3'-5' Exonuclease Activity and Assessment of dUTP Incorporation by PCR

There are several methods of measuring 3' to 5' exonuclease activity known in the art, including that of Kong et al. (Kong et al., 1993, J. Biol. Chem. 268: 1965) and that of Southworth et al. (Southworth et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93: 5281), the full contents of both of which are hereby incorporated by reference. For example, the exonuclease activity of wild type and active JDF-3 mutant polymerases as measured by the Kong et al. method were as follows: (other DNA polymerase mutants may be measured similarly)

Exo activity (U/mg):	
Wt	915
D141A	7
D141N	953
D141S	954
D141T	0.5
D141E	940
E143A	0.3

Partially-purified mutant preparations (heat-treated bacterial extracts) are assayed for dUTP incorporation during PCR. For example, a 2.3kb fragment containing the Pfu pol gene was from plasmid DNA using PCR primers: (FPfuLIC) 5'-gACgACgACAAgATgATTTTAgATgTggAT-3' (SEQ ID NO:1) and (RPfuLIC) 5'-ggAACAAgACCCgTCTAggATTTTTTAATg-3' (SEQ ID NO: 2). Amplification reactions

consisted of 1x cloned Pfu PCR buffer, 7 ng plasmid DNA, 100ng of each primer, 2.5U of Pfu mutant (or wild type Pfu), and 200μM each dGTP, dCTP, and dATP. To assess relative dUTP incorporation, various amounts of dUTP (0-400μM) and/or TTP (0-200μM) were added to the PCR reaction cocktail. The amplification reactions were cycled as described in example 6. Other DNA polymerase mutants may be similarly tested.

Partially-purified preparations of the V93E and V93R mutants showed improved dUTP incorporation compared to wild type Pfu (Figure 2a). Each mutant successfully amplified a 2.3kb target in the presence of 200μM dUTP (plus 200μM each TTP, dATP, dCTP, dGTP). In contrast, extracts containing the Pfu V93N, V93G, V93H, and V93L mutants showed little-to-no amplification in the presence of 200μM dUTP, similar to wild type Pfu (data not shown). Additional testing showed that the Pfu V93R mutant extract amplified the 2.3kb target in the presence of 100% dUTP (0% TTP)(Figure 2b).

KOD: Partially-purified preparations of KOD V93D, E, K, Q, and R showed reduced uracil sensitivity as evidenced by successful amplification of the 970bp amplicon using dU-containing primers and TTP (Figure 11). In contrast, wild type KOD and the KOD V93N mutant were unable to amplify using dU-primers and TTP. Only the KOD V93K and V93R mutants showed complete or nearly complete elimination of uracil sensitivity as shown by successful amplification in the presence of 100% dUTP (Figure 11). In contrast, the KOD V93D, E, and Q substitutions only partially reduce uracil sensitivity since these mutants are unable to amplify in the presence of 100% dUTP.

The rationale for determining relative uracil sensitivity using PCR assays is as follows. Successful amplification with dU-primers indicates that reduction in uracil sensitivity is sufficient to allow the mutants to polymerize past the nine uracils in the PCR primers (to create the primer annealing sites). However, mutants that successfully amplify in the presence of 100% dUTP, must lack or almost completely lack uracil sensitivity, since they must polymerize past numerous uracils (~230 uracils per strand; 925bp segment synthesized with 25% T content) in the template strand.

Tgo: Only the *Tgo* V93R mutant successfully amplified the 0.97kb amplicon in the presence of 100% dUTP (Figure 12), indicating that the arginine substitution was most effective in reducing uracil sensitivity.

JDF-3: Only the JDF-3 V93R and V93K mutants successfully amplified the 0.97kb amplicon in the presence of 100% dUTP (Figure 12), indicating that the arginine and lysine substitutions were the most effective in reducing uracil sensitivity. Product yields with 100% dUTP were noticeably lower than yields with 100% TTP suggesting that in JDF-3, the V93R mutation does not completely eliminate uracil sensitivity (Figure 13). In contrast, Pfu V93R, *Tgo* V93R, and KOD V93R produce similar yields with TTP and dUTP, indicating that uracil sensitivity is almost completely eliminated.

Pfu deletions. We constructed deletions (92,92,94, 92-93, 93-94, 92-94) and insertions (1-3 glycines between D92 and V93) in Pfu centering around V93. Only the Pfu delta V93 and delta D92-V93-P94 mutants showed a reduction in uracil sensitivity (Figure 14). Based on amplification of 0.6kb, 2.6kb, and 6kb genomic amplicons, relative uracil sensitivity was determined as follows: (least sensitive/highest dTUP incorporation) Pfu V93R > Pfu delta 93 > Pfu delta 92-94 > wild type Pfu (most sensitive/no dUTP incorporation).

Example 4. Purification of DNA Polymerase Mutants

Bacterial expression of Pfu mutants. Pfu mutants (Tgo, or KOD or JDF-3 mutants) can be purified as described in US 5,489,523 (purification of the exo$^-$ Pfu D141A/E143A DNA polymerase mutant) or as follows. Clarified, heat-treated bacterial extracts were chromatographed on a Q-Sepharose™ Fast Flow column (~20ml column), equilibrated in buffer B (buffer A plus 0.1% (v/v) Igepal CA-630, and 0.1% (v/v) Tween 20). Flow-through fractions were collected and then loaded directly onto a P11 Phosphocellulose column (~20ml), equilibrated in buffer C (same as buffer B, except pH 7.5). The column was washed and then eluted with a 0-0.7M KCl gradient/Buffer C. Fractions containing Pfu DNA polymerase mutants (95kD by SDS-PAGE) were dialyzed overnight against buffer D (50mM Tris HCl (pH 7.5), 5mM βME, 5% (v/v) glycerol, 0.2% (v/v) Igepal CA-630, 0.2% (v/v) Tween 20, and 0.5M NaCl) and then applied to a Hydroxyapatite column (~5ml), equilibrated in buffer D. The column was washed and Pfu DNA polymerase mutants were eluted with

buffer D2 containing 400 mM KPO_4, (pH 7.5), 5mM βME, 5% (v/v) glycerol, 0.2% (v/v) Igepal CA-630, 0.2% (v/v) Tween 20, and 0.5 M NaCl. Purified proteins were spin concentrated using Centricon YM30 devices, and exchanged into Pfu final dialysis buffer (50mM Tris-HCl (pH 8.2), 0.1mM EDTA, 1mM dithiothreitol (DTT), 50% (v/v) glycerol, 0.1% (v/v) Igepal CA-630, and 0.1% (v/v) Tween 20).

Protein samples were evaluated for size, purity, and approximate concentration by SDS-PAGE using Tris-Glycine 4-20% acrylamide gradient gels. Gels were stained with silver stain or Sypro Orange (Molecular Probes). Protein concentration was determined relative to a BSA standard (Pierce) using the BCA assay (Pierce).

Results: Pfu exo- D141A/E143A mutants with additional V93E or V93R mutations were purified to ~90% purity as determined by SDS-PAGE.

Example 5. Determining Mutant Polymerase Unit Concentration and Specific Activity

The unit concentration of purified Pfu mutant preparations was determined by PCR. In this assay, a 500bp *lac*Z target is amplified from transgenic mouse genomic DNA using the forward primer: 5'-GACAGTCACTCCGGCCCG-3' (SEQ ID NO:15) and the reverse primer: 5'-CGACGACTCGTGGAGCCC-3' (SEQ ID NO: 16). Amplification reactions consisted of 1x cloned Pfu PCR buffer, 100ng genomic DNA, 150ng each primer, 200μM each dNTP, and varying amounts of either wild type Pfu (1.25U to 5U) or Pfu mutant (0.625-12.5U). Amplification was performed using a RoboCycler® temperature cycler (Stratagene) with the following program: (1 cycle) 95°C for 2 minute; (30 cycles) 95°C for 1 minute, 58°C for 1 minute, 72°C for 1.5 minutes; (1 cycle) 72°C for 7 minutes. PCR products were examined on 1% agarose gels containing ethidium bromide.

Results: Figure 3 contains a table listing the protein concentration, unit concentration, and specific activity of the purified Pfu V93R and V93E mutants.

The purified mutants were also re-assayed to assess dUTP incorporation during PCR, according to the method described in Example 3. Figure 4 shows that the Pfu V93R mutant produces similar yields of the 500bp amplicon in the presence of 100% TTP (lane 8), 50%

TTP:50% dUTP (lane 5), and 100% dUTP (lane 7), while the Pfu V93E mutant produces high yields in the presence of 100% TTP (lane 1) and 50% TTP:50% dUTP (lane 3) and lower yields in the presence of 100% dUTP (lane 4). In contrast, cloned Pfu can only amplify in the presence of 100% TTP (lane 12). These results indicate that the V93R and V93E mutations significantly improve dUTP incorporation compared to wild type Pfu, and that the V93R mutation appear to be superior to the V93E mutation with respect to reducing uracil detection.

Example 6. PCR Amplification with Purified DNA Polymerase Mutants

PCR reactions are conducted under standard conditions in cloned Pfu PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris HCl (pH 8.8), 2mM Mg SO_4, 0.1% Triton X-100, and 100μg/ml BSA) with various amounts of cloned Pfu, Pfu*Turbo*, or mutant Pfu DNA polymerase. For genomic targets 0.3-9kb in length, PCR reactions contained 100ng of human genomic DNA, 200μM each dNTP, and 100ng of each primer. For genomic targets >9kb in length, PCR reactions contained 250ng of human genomic DNA, 500μM each dNTP, and 200ng of each primer.

Table 3 - Cycling Conditions:

Table 4

Amplicon	PCR primers	Cycling conditions
0.6kb lambda	F: 5'-GGAATGAAGTTATCCCCGCTTCCCC (SEQ ID NO: 41) R: 5'-CCAGTTCATTCAGCGTATTCAG-3' (SEQ ID NO: 42)	• 93°C 1 min (1x) • 93°C 1 min, 60°C 40s, 72°C 1 min (30x) • 72°C 10 min (1x)

0.97 lambda	FU: 5'-GGAAUGAAGUUAUCCCCGCUUCCCC- (SEQ ID NO: 75) RU: 5'-CCAGGUCUCCAGCGUGCCCA-3' (SEQ ID NO: 76) FT: 5'-GGAATGAAGTTATCCCCGCTTCCCC (SEQ ID NO: 77) RT: 5'-CCAGGTCTCCAGCGTGCCCA-3' (SEQ ID NO: 78)	• 93°C 1 min (1x) • 93°C 1 min, 60°C 50s, 72°C 1 min (30x) • 72°C 10 min (1x)
2.6kb Human genomic (α1 anti-trypsin)	F: 5'GAG GAG AGC AGG AAA GGT GGA AC (SEQ ID NO: 79) R: 5'TGC AGA GCG ATT ATT CAG GAA TGC (SEQ ID NO: 80)	• 95°C 2 min (1x) • 95°C 40s, 58°C 30s, 72°C 3 min (30x) • 72°C 7 min (1x)
6kb Human genomic (α1 anti-trypsin)	F: 5'GAG GAG AGC AGG AAA GGT GGA AC (SEQ ID NO: 81) R: 5' GAG CAA TGG TCA AAG TCA ACG TCA TCC ACA GC (SEQ ID NO: 82)	• 92°C 2 min (1x) • 92°C 10s, 58°C 30s, 68°C 12 min (10x) • 92°C 10s, 58°C 30s, 68°C 12 min plus 10s/cycle (20x) • 68°C 10 min (1x)

Pfu mutants are described here as examples, but the same protocol can be used for PCR by other DNA polymerase mutants (e.g., KOD and JDF-3). Comparisons were carried out to determine if mutations that improve dUTP incorporation, and hence reduce uracil detection, also improve PCR performance. In Figure 5, a 12kb target was amplified from human genomic DNA using 2 min per kb extension times. Under these conditions, 1U, 2U, and 4U of the Pfu V93R mutant successfully amplified the target, while the same amount of cloned Pfu could not. In comparison, Pfu*Turbo* successfully amplified the long target; however, PCR product yields were significantly lower than those produced with the V93R mutant (Figure 5). Similar experiments employing 1 min per kb extension times showed that the 12kb target could be amplified in high yield with 5U and 10U of Pfu V93R and amplified in low yield with 10U of Pfu*Turbo* (data not shown). In total, these results demonstrate that the V93R mutation dramatically improves the PCR performance of Pfu DNA polymerase.

Similar testing of the purified Pfu V93E mutant showed that although the V93E mutation improves dUTP incorporation (Figure 2), this mutant is not robust enough to amplify the long 12kb amplicon when assayed using enzyme amounts between 0.6U and 10U (data not shown). In comparison, the product was successfully amplified using 10U of Pfu*Turbo* (data not shown).

Figure 8 shows the results of additional Pfu mutations on dUTP incorporation. Pfu V93K and V93R mutants show significantly improved dUTP incorporation compared to wild type Pfu. In contrast, the Pfu V93W, V93 V93W, V93Y and V93M mutants showed little to no improvement in dUTP incorporation (see Figure 8A). In addition, both V93D and V93R mutants showed significantly improved dUTP incorporation, compared to wild type (Figure 8B), while the V93N mutation showed a very small improvement in dTUP incorporation (Figure 8C). The Pfu V93G mutation showed little to no improvement in dUTP incorporation.

Example 7 Construction of Pfu DNA Polymerase Deletion and Insertion Mutants

Mutants with altered polymerization activity may also be constructed using the exo- and/or V93 mutants obtained. For example, insertions and deletions were introduced in Pfu DNA polymerase in the region around V93 using the QuikChange Multi Site-Directed

Mutagenesis Kit (Stratagene). Figure 10 lists the primer sequences employed to generate useful mutations. Clones were sequenced to identify the incorporated mutations.

The following Pfu mutants were constructed: deletions of residues 93, 92, 94, 92-93, 93-94, and 92-94, and insertions of one, two, or three glycines between residues 92 and 93.

Example 8 Quantitative PCR Using Mutant DNA Polymerase of the Present Invention

PCR reactions may be set up as described above in Example 6. A Taqman probe (labeled) may be added as described by Applied Biosystems (CA). an oligonucleotide probe containing a reporter molecule - quencher molecule pair that specifically anneals to a region of a target polynucleotide "downstream", i.e. in the direction of extension of primer binding sites. The reporter molecule and quencher molecule are positioned on the probe sufficiently close to each other such that whenever the reporter molecule is excited, the energy of the excited state nonradiatively transfers to the quencher molecule where it either dissipates nonradiatively or is emitted at a different emission frequency than that of the reporter molecule. During strand extension by a mutant DNA polymerase of the present invention, the probe anneals to the template where it is digested by the 5' to 3' exonuclease activity of the polymerase. As a result of the probe being digested, the reporter molecule is effectively separated from the quencher molecule such that the quencher molecule is no longer close enough to the reporter molecule to quench the reporter molecule's fluorescence. Thus, as more and more probes are digested during amplification, the number of reporter molecules in solution increases, thus resulting in an increasing number of unquenched reporter molecules which produce a stronger and stronger fluorescent signal.

are labeled with a fluorophore and a quencher of that fluorophore, respectively. In the absence of target polynucleotide, the complementary sequences on either end of the molecule permit stem formation, bringing the labeled ends of the molecule together, so that fluorescence from the fluorophore is quenched. In the presence of the target polynucleotide, which bears sequence complementary to the loop and part of the stem structure of the beacon probe, the intermolecular hybridization of the probe to the target is energetically favored over intramolecular stem-loop formation, resulting in the separation of the fluorophore and the quencher, so that fluorescent signal is emitted upon excitation of the fluorophore. The more target present, the more probe hybridizes to it, and the more fluorophore is freed from quenching, providing a read out of the amplification process in real time.

All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.